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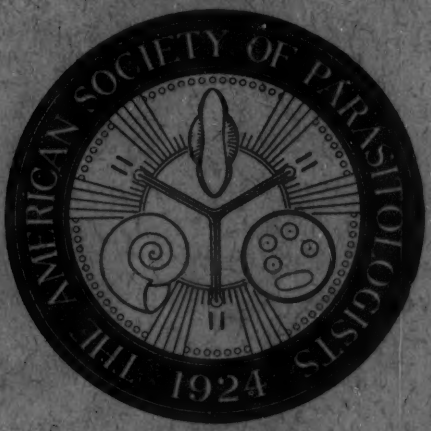

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DIFFERENCES IN STRAINS OF *TRICHOMONAS VAGINALIS* AS REVEALED BY INTRAPERITONEAL INJECTIONS INTO MICE*

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Recent literature has indicated the value of the mouse as an experimental animal for *Trichomonas vaginalis*. Lesions due to the parasite have resulted following injection by subcutaneous, intramuscular, and intraperitoneal routes (Schnitzer et al, 1950, and Iwai, 1957); in the latter case, fatal infections have also developed (Hamada, 1953, 1956, and Inoki and Hamada, 1953). The mouse has also provided evidence for differences in the virulence of strains of *T. vaginalis* (Honigberg and Braunthal, 1957, and Bogovsky and Teras, 1958), and has been used as a tool for assaying trichomonacidal activity of drugs (Hamada, 1955; Lynch et al, 1955; and Vaisman and Hamelin, 1958).

In this laboratory, a series of investigations was carried out several years ago to assess the importance of *T. vaginalis* as a human pathogen and to relate its presence to cytological changes in the vaginal epithelium, as well as to study the epidemiology and treatment of trichomoniasis (Burch et al, 1958, 1959a, and 1959b). One of the most striking observations was the different degree of inflammation found in women with trichomoniasis. It was considered that these variations could be due, at least in part, to differences in the pathogenetic potentialities of different strains of the parasite. The studies to be described below were initiated with the aims of (1) determining whether or not a relation exists between the ability of a strain to cause lesions in mice and the pathology observed in the patient from whom the strain was derived, (2) to ascertain if intraperitoneal infections in mice could be standardized sufficiently to provide a tool for chemotherapeutic studies, and (3) to investigate possible differences in the susceptibility of mouse strains to *T. vaginalis*. Studies on differences in strains of *T. vaginalis* have been carried out at N.I.H. in germ-free and in conventional guinea pigs (Newton et al, 1960).

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MATERIAL AND METHODS

Thirteen strains of *T. vaginalis* were studied. These strains were derived from patients in the NIH study (Burch et al, 1958) and were designated B, E-BR, C-1, C-2, H-1, H-2, La, Lo, Log, N, R, W-ur, and W-vag. Strains C-2, H-2, and W-vag came from the same patients as C-1, H-1, and W-ur, but were isolated about 1 year later. All the strains came from the vagina, except W-ur from the urethra and Lo from the urine; Lo was from a male patient. When first used, most of the strains had been in bacteria-free cultivation for about 1 year (8 to 18 months); however, R was 2 years old, and N, La, and B had been in cultivation for 3 to 6 months. The symptomatology associated with particular strains will be discussed briefly below.

The trichomonads were isolated and maintained in trypticase-liver-serum (TLS) medium into which penicillin and streptomycin (50 units and micrograms respectively per ml) were used until the cultures were free of bacteria (Burch et al, 1959a). To obtain concentrated material for injection, the organisms were grown in 250-ml centrifuge bottles, in some cases in 125-ml Erlenmeyer flasks, at 35 C for 48 to 72 hours. Usually 4 bottles were prepared for each strain used. After incubation the cultures were centrifuged at 2000 rpm for 10 to 15 minutes. The supernatant fluid was drawn off aseptically and the sediments resuspended in 1 to 5 ml of 0.85 percent saline. Tests for bacterial sterility of the harvests were made by examination of Gram-stained smears and inoculation of blood agar plates and fluid thioglycolate

medium which were examined for growth after 3 and 10 days, respectively; tests for virus and PPLO organisms were also carried out.

Counts of the flagellates in the pooled harvests were made following a 1:100 dilution in trypticase-liver medium containing about 1.0 percent formalin to immobilize the trichomonads. A Neubauer-Levy hemocytometer was used and counts were made by two persons, each reading 1 sq mm of the chamber. The harvest was then adjusted with sterile medium or with 0.85 percent saline solution to furnish a suspension containing the desired number of organisms for injection into mice. The inoculum per mouse consisted of 0.5 ml of the suspension and contained 1×10^5, 1×10^6, and 1×10^7 organisms in experiments where graded inocula were used, and 1×10^7 in all other experiments. Male mice were used throughout and were injected intraperitoneally, usually in lots of 10 for each experimental group. The age of the mice used is given under "Results." The mice were housed in plastic cages, five per cage, with food and water supplied ad libitum. They were observed daily following inoculation, and the time of death was noted. When they appeared moribund or died, observations were made of the gross pathology, and in the case of one virulent strain (C-1) tissues were preserved for histopathologic study.

The strains of mouse used in the experiments were furnished by the Animal Production Section of NIH and were: A/LN, C57BL, A/HeN (inbred strains), C3H/p, CFW (brother-sister matings) and GP (general purpose random matings).

In assaying the comparative virluence of trichomonad strains, the C-1 strain served as the standard, and A/LN mice, of comparable age, were used throughout. Because the time required for the various preparations and manipulations made it impractical to compare all strains of *Trichomonas* at the same time, from 1 to 4 strains were tested against the standard C-1 strain in a single run.

In each test of the susceptibility of mouse strains, the C-1 and R strains of *T. vaginalis* were used. The A/LN mouse was the standard and one or more additional strains were compared to it. As all mouse strains of comparable age could not be obtained at the same time, the various strains were therefore tested against A/LN rather than against each other.

Since it was not possible to compare all the strains of parasites or all the strains of mice at the same time, and since the large number of small experiments were difficult to interpret separately, the results of the experiments were combined for purposes of presentation. The small differences in the age of the mice at inoculation, the differences in the ages at which the living mice were sacrificed and the differences in time in which the experiments were conducted make combined results questionable. However, the consistency of the results obtained with the C-1 strain of parasites and the A/LN strain of mice in the various experiments indicates that the effects of the differences between experiments are small compared with some of the factors of major interest. While these considerations make significance tests of doubtful value, the differences are not large enough to necessitate the presentation of each experiment separately.

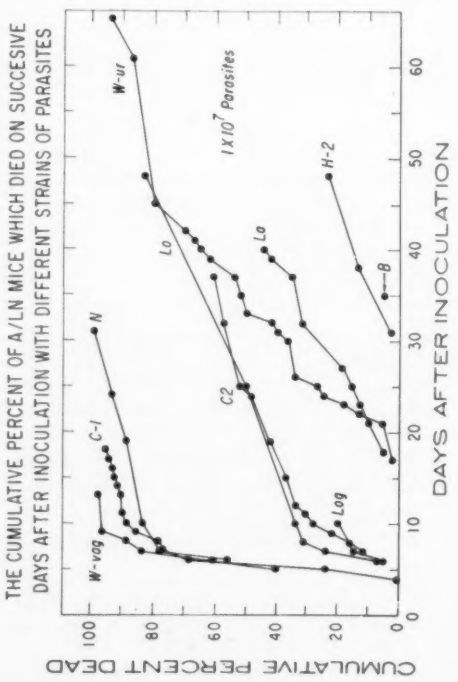
The method of combining results was the standard life table technique (Cutler and Ederer, 1958). The cumulative percent dead was calculated for each day after inoculation. In this way the mice which died or were sacrificed were removed from the calculation of the figure for each successive day and it should be remembered that the curves in figures 1 and 2 are based on smaller numbers of mice as the number of days after inoculation increases.

RESULTS

Protozoan Strain Differences

The first test involved a comparison between 2 strains of *T. vaginalis,* one derived from a case of severe vaginitis (C-1) and one from a mild case (R). With these 2 strains there was a pronounced difference in virulence. With C-1, inocula of 1×10^7 parasites regularly produced fatal infections in mice. In 10 experiments, a total of 96 mice was injected, of which 93 succumbed to the infection within relatively short

FIGURE 1.



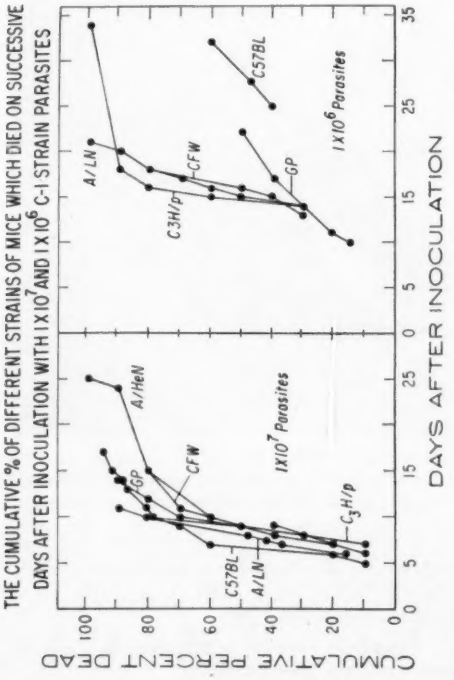
periods of time with a combined median day of death 6.7. On the other hand, comparable inocula of the R strain never produced fatalities; at the most, minimal lesions were observed. Inocula of 1×10^6 C-1 parasites also produced death in mice; here the survival time was extended up to 3 weeks. In one experiment in which 1×10^6 parasites were inoculated into A/LN mice, the median day of death was 15.5 as compared with 8.5 for 1×10^7. Inocula of 1×10^5 organisms of C-1 strain failed to kill mice.

TABLE I. *Combined results of 15 experiments comparing various* Trichomonas vaginalis *strains against the C-1 strain in A/LN mice.**

Strain of parasite	No. of experiments	Total no. of mice inoculated	Total no. dead
C-1	15	148	143
C-2	4	32	18
Lo	3	30	10
W-ur	4	40	34
La	4	40	16
W-vag	4	40	39
N	2	18	18
E-Br	2	20	0
H-1	4	40	0
H-2	3	30	4
B	2	20	1
Log	2	20	4

* Age of mice was the same in each experiment and varied from 32–38 days.

FIGURE 2.



For experiments on comparative pathogenicity of other strains of *T. vaginalis*, the 1×10^7 inoculum was used. The data of 15 experiments have been combined and are presented in table I and figure 1. The data show clearly that strains C-1, W-vag, and N are highly virulent, killing the majority of mice in relatively short periods. Strains H-1, H-2, B, and E-BR appear to have minimal virulence (with E-BR, and H-1 not producing fatalities and therefore, not cited in figure 1). The remaining strains, C-2, Lo, Log, W-ur, and La, appear intermediate in virulence, either failing to kill the majority of mice or doing so only after prolonged periods.

In strains arising from the same patient two noteworthy differences are evident from the data. With the C and W strains there appears considerable difference in virulence from one isolation to another whereas the H strains appear similar with minimal or no virulence in either. The possible reasons for these differences will be discussed below.

Thus the data show that we have encountered a wide range of virulence among the strains tested.

Susceptibility of Strains of Mice

The combined data from five experiments comparing strains of mice as to their susceptibility to infection with *T. vaginalis* are given in table II and figure 2. The data from mice inoculated with 1×10^7 parasites present no striking differences among the strains of mice employed, all appearing equally susceptible to the infection. In mice inoculated with 1×10^6 trichomonads there appears to be some variabil-

TABLE II. *Comparison of the susceptibility of strains of mice to* T. vaginalis *(C-1 strain).*

No. of parasites	Strain of mice	No. of experiments	Total mice inoculated	Total no. dead
1×10^7	A/LN	5	49	43
	GP	1	10	9
	C3H/p	1	10	10
	C57BL	1	10	8
	CFW	1	10	8
	A/HeN	1	10	10
1×10^6	A/LN	1	10	10
	GP	1	10	5
	C3H/p	1	10	10
	C57BL	1	5	3
	CFW	1	10	8

ity in that General Purpose mice and C57BL mice showed fewer deaths from the inoculation than did the other strains.

Some tests were also performed with the R strain of *T. vaginalis* using the same strains of mice none of which was found particularly vulnerable to this trichomonad of low virulence.

FIGURE 3. Lesions (indicated by arrows) produced by R strain of *Trichomonas vaginalis*. These represent maximum lesions produced by this strain.

FIGURE 4. Lesion produced by C-1 strain of *Trichomonas vaginalis*. Large areas of necrosis of the liver are indicated by the arrow.

Pathology

In mice infected intraperitoneally with a *T. vaginalis* strain of low virulence (R), the lesions when present were minimal. They consisted mainly of caseous masses adherent to the peritoneum and contained trichomonads (fig. 3). In mice with severe infections resulting from a virulent strain, C-1, the caseous masses if present were usually much larger. In addition, there was inflammatory involvement, often severe, of mesentery, omentum, pancreas, perirenal tissue, and liver. In the liver extensive necrosis frequently occurred (fig. 4.) In this organ, invasion took place most frequently at the hilus, the process proceeding distally in a wave-like manner, leaving behind necrotic parenchyma and inflamed, but intact, major portal triads (fig. 5). At the forward edge of the process there was always a band of parasites and just proximal to this band there was a zone of infiltrating, inflammatory cells, principally polymorphonuclear leucocytes.

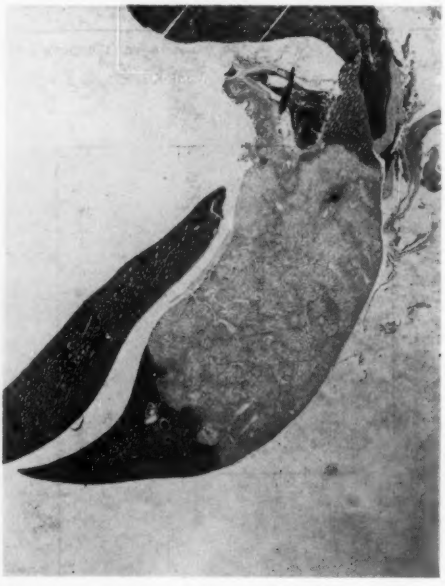


FIGURE 5. Necrosis of liver proceeding distally from hilus in C-1 strain of *Trichomonas vaginalis*. Only the tip of the lobe shows normal parenchyma.

Pathologic changes, in greater detail, will be presented in a later paper.

DISCUSSION

The differences among strains of *T. vaginalis* observed in these experimental studies in mice can be assumed, in part, to reflect variations in the virulence of these organisms. Honigberg and Braunthal (1957) stated that the differences among three strains of *T. vaginalis* on ability to produce subcutaneous abscesses in mice "reflect the actual differences in virulence of the strains in man." Likewise Bogovsky and Teras (1958) reported differences in virulence of strains of *T. vaginalis* in mice. They too noted that a definite correlation existed between the clinical condition of colpitis in patients examined and the pathogenicity in mice. It is a little more difficult, in appraising our strains, to be as definite in such a conclusion. All of the patients from whom the strains were isolated had bouts of vaginal or urethral inflammation. The strains (C-1 and W-vag) which appeared to have similar high virulence for mice were derived from severe cases of vaginitis, and strain R was taken from a patient with very mild inflammation. Other strains (C-2, Lo, N and W-ur) which appeared intermediate in virulence for mice, were from both mild and severe cases. Our data therefore may indicate some correlation between pathogenicity in the two hosts, but because of the multiple factors involved in susceptibility, the degree of pathology seen in a particular patient must be correlated with the resistance of the individual as well as the virulence of the strain.

Variation in the virulence of strains isolated from the same patient on different occasions have been described above. There are at least 3 possible explanations of these findings: (1) The isolation may have been of different strains, representing re-infections of the patient; (2) the strains may have been modified as the result of therapy; and (3) the strains may have become modified as the result of a host response. At the present stage of our knowledge, it is possible to do nothing more than conjecture about the choice of these explanations.

A discussion as to whether or not the slight differences in suspectibility of mouse strains to *T. vaginalis* represent constant differences must await replicate tests of the strains. It is possible that greater differences may become apparent with the use of a strain of *T. vaginalis* of moderate rather than high virulence.

It appears feasible to use the intraperitoneal infection in mice as a standard tool in chemotherapeutic studies. The results with the C-1 strain in A/LN mice, shown above, have been quite consistent. Thus, it should be possible to use this system and to test drugs given orally or parenterally for the effectiveness in clearing trichomonads from the animal. The search for a systemic drug against *T. vaginalis* would thus be facilitated.

SUMMARY

Thirteen strains of *T. vaginalis* derived from human infections of varying severity were tested by intraperitoneal injection into mice to reveal possible differences. One strain, C-1, derived from a severe case of vaginitis, regularly produced death in mice when injected in dosages of 1×10^6 or higher. Another, the R strain, from a mild infection, failed to produce death in mice in parallel tests. Strains from other sources with moderate symptomatology, did not reveal such clear-cut differences. Strains isolated from the same individual on different occasions also showed differences. Various strains of mouse were tested for susceptibility to *T. vaginalis:* A/LN, C57BL, A/HeN (inbred strains); C3H/p, CFW (brother-sister matings); and GP (general purpose random matings). All were similar except for a possibly slightly greater susceptibility in the case of the A/LN strain. It is suggested that the regularity of death of mice injected with the C-1 strain parasites could be useful in chemotherapeutic trials.

ACKNOWLEDGMENTS

The authors wish to express appreciation to Doctor L. B. Crittenden and to Mr. Max Myers of the Biometry Branch of the National Cancer Institute for preparation of graphs and assistance in presentation of data.

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RESEARCH NOTE

OCCURRENCE OF *TRICHOMONAS GALLINAE* IN THE GROUND DOVE, *COLUMBIGALLINA PASSERINA* (L.).

In July 1960, field studies were conducted in the vicinity of McAllen, Texas, to determine the status of trichomonad infection among the large population of mourning and white-winged doves that nest in the desert shrub of the Lower Rio Grande Valley. During the course of this work a small series of nestling ground doves, *Columbigallina passerina* (L.), also was examined for trichomonad infection. On July 13 two sibling ground doves, each about 1 week old, were located in a nest on the Pharr Settling Basin, 6 miles south of McAllen. Saliva was collected from each bird and inoculated into tubes of LBSS culture medium. The inoculated tubes were mailed to the Patuxent Wildlife Research Center for examination. Both tubes contained active *Trichomonas gallinae* when examined at the laboratory on July 16.

A second pair of ground-dove nestlings, 10 to 12 days old, was examined July 22; only one of the pair was found to be infected with *T. gallinae*. None of the 3 infected nestlings showed any gross lesions. This is to the best of our knowledge the first report of *T. gallinae* from this species of dove.

We wish to thank Mr. William Frazer, Texas Game and Fish Commission, for directing us to the Pharr Settling Basin area where these studies were made.—LOUIS N. LOCKE, FRANCES S. LOCKE AND DAVID H. REESE, *Patuxent Wildlife Research Center, Laurel, Maryland.*

INDUCED RESISTANCE OF MICE TO SUBCUTANEOUS INFECTION WITH *TRICHOMONAS GALLINAE* (RIVOLTA, 1878)*

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Honigberg and Braunthal (1957) had reported that virulence of strains of *Trichomonas gallinae* and *T. vaginalis* grown in vitro could be evaluated by producing lesions in mice at the site of subcutaneous inoculations. These observations were utilized to commence studies on the ability of excretions or secretions of the trichomonads to induce a protective response against challenge subcutaneous infections in white mice.

In order to carry out the immunization studies it was necessary to obtain basic information on the model infection employed. Therefore, the following factors were analyzed: (1) the degree of susceptibility of the strains of mice used; (2) the size and type of infecting inoculum needed for inducing lesions; and (3) the macro- and microscopic anatomy of the lesions.

MATERIALS AND METHODS

The strain of *T. gallinae* used in this study was derived from cultures of the Jones' barn strain (Stabler, 1948a; 1953) kindly supplied by Dr. B. Honigberg, and reisolated from a subcutaneous lesion in a mouse. Axenic stock cultures were maintained in Brewer's thioglycollate medium (Baltimore Biological Laboratory) supplemented with 4 percent horse serum. These cultures were routinely passaged approximately every 48 hours by transferring 0.05 ml of the 48-hour culture to fresh medium.

Animals used in this study were either mature female Holtzman white mice ca 35 g body weight or mature female Rockland Swiss Webster strain white mice ca 25 g body weight. Animals were always maintained on Purina laboratory chow.

The inoculum used for inducing subcutaneous lesions in mice was prepared in the following manner. Using sterile technique throughout, a twice passaged 48-hour stock culture of *T. gallinae* was centrifuged at 1000 *g* for 10 minutes, and then a portion of the supernatant was removed to adjust the concentration of organisms to a level of 5×10^6 per ml. Two tenths ml of the concentrate was inoculated subcutaneously into the shaven hind right thigh of a white mouse. Lesion size was determined by measuring the maximum diameter with a millimeter rule.

Protective antigens were prepared in the following manner: 0.5 ml of twice passaged 48-hour stock cultures were transferred to tube cultures of cysteine-peptone-liver-maltose (CPLM) medium without agar plus 4 percent horse serum (Johnson and Trussell, 1943). A 24-hour tube culture was used to inoculate 500 ml of CPLM medium in prescription bottles. After 23 hours the bottle cultures were harvested by centrifugation at 1000 *g*, and thrice washed with Krebs-Ringer solution buffered with 0.01 M phosphate buffer (KRP) at pH 6.8. The organisms were then resuspended in 30 ml of saline-trypticase (ST) medium of Kupferberg et al (1948) without serum and agar. Ten ml of the latter suspension were transferred to each of three 250 ml Erlenmeyer flasks containing 25 ml of ST medium. One ml of the final suspension was taken from each flask as an aliquot for hemacytometer counts in order to determine the initial concentration of organisms per flask. The suspensions were then incubated in a water bath at 37 C, while being shaken at 60 oscillations per minute, for a period of 4 hours. After 4 hours an aliquot of 1.0 ml was taken from each flask to check the concentration of organisms. The suspensions were cooled in an ice bath, centrifuged at 1000 *g* for 20 minutes at 5 C. The supernatant was decanted off and filtered through a millipore HA white plain 047 mm filter (Millipore Filter Corp., Bedford, Mass.), and lyophilized. The lyophilized antigen was stored in a vial at − 20 C until used for immunization experiments. ST medium without organisms was similarly treated, and served as the medium control in immunization experiments. The pellett was thrice washed with KRP and stored at − 20 C until used as an organism control antigen.

For histological studies lesions were carefully excised intact with a minimum of surrounding tissue, and then fixed in Bouin's fixative. After fixation the lesions were infiltrated with paraffin, sectioned, and stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

Susceptibility to Infection

Two hundred untreated mice received subcutaneous thigh inoculations of *T. gallinae*. All of 85 Holtzman mice were susceptible to infection. Of 119 Rockland Swiss Webster mice infected, only 4 were resistant to infection. Thus we have concluded that essentially both strains

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of mice are equally susceptible to the infection.

The Infecting Inoculum

It was found that 48-hour cultures were best suited for the production of lesions. One million organisms would produce a lesion, and this quantity could be conveniently obtained in 0.1 to 0.2 ml quantities of concentrated medium from a 48-hour culture. No significant variation was noted if the culture age was increased to 56 hours.

The Nature of the Lesions

Both the constituents of the thioglycollate medium and the trichomonads are necessary to produce lesions. Organisms in medium without agar do not produce a lesion. Agar will not produce lesions without organisms present. If an inoculum of 1×10^6 (or greater) organisms in thioglycholate medium was administered, lesions appeared as turgid swellings between the fourth and eighth day after inoculation, but rarely after the seventh day. However, when the infectious dose was reduced 50 percent (5×10^5) the appearance of lesions was delayed considerably; the time of appearance being as late as the twelfth day after inoculation (see table I).

TABLE I.

Relationship between number of organisms administered and time of appearance of lesion.

Inoculum No. organisms	Number of determinations	Mean time of appearance in days	Range
10^6	17	5.3	4–8
5×10^5	6	10.6	10–12

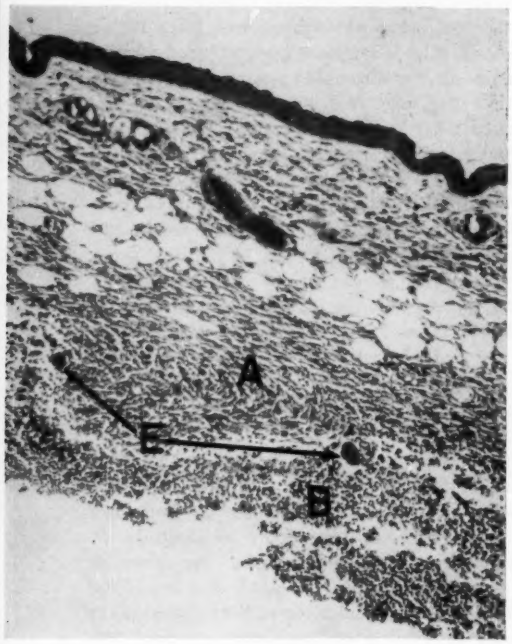
Data on lesion size indicates that a lesion approximately 1.0 cm in diameter develops by the fifth to sixth day after inoculation. The fifth to sixth day was used as the criterion for analyzing effects of immunization because a large number of lesions would ulcerate externally and drain between the sixth and eighth days after inoculation. Occasionally lesions would develop more rapidly and drain by the fifth or sixth day. Thus the fate of the lesions after the sixth or seventh day is extremely uncertain, and its status cannot be reliably compared from one animal to another during these later stages of infection.

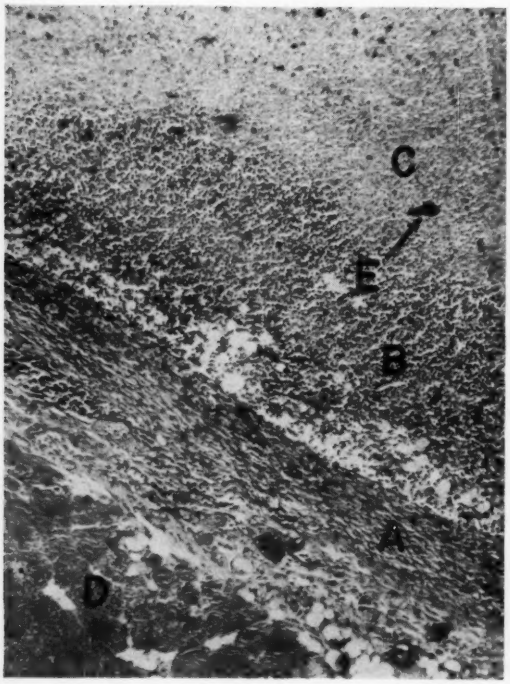
Microscopic analysis of the lesions formed in untreated animals reveals an interesting picture. Histologically the lesions consist of three zones: (a) an inner core consisting of caseous material containing many degenerate trichomonads, polymorphonuclear leukocytes, a few mononuclear leukocytes, and residual agar remaining from the original inoculum; (b) a medium zone of living trichomonads with some white cells interspersed throughout this band; (c) and an outer core consisting of a thick growth of dense connective tissue which in appearance suggests a derivation from the reticular layer of the derma. The reticular layer has thickened sufficiently to all but obliterate the papillary layer. This thick connective tissue layer surrounds the lesion on all sides except where pressure necrosis has brought about an ulceration of this connective tissue capsule. The connective tissue capsule displaces the bulk of the hypodermal layer of the skin, and the connective tissue layer extends down to the underlying musculature. The agar appears to serve as an irritant which induces formation of the connective tissue capsule. Within this capsule the trichomonads grow in a virtual in vivo "test tube." Typical lesions 5 days after infection are shown in figure 1.

Results of artificial immunization with physiologically derived (PD) antigens

On the basis of cell counts made on 6-day-old lesions 350×10^6 *T. gallinae* were incubated in 100 ml ST medium for a period of 4 hours yielding 1.4×10^9 trichomonad-hours units of antigen (1 unit equals 1 trichomonad $\times$ 1-hour incubation.) The organisms were sedimented from the medium and the supernatant was lyophilized and stored dry until used for immunization experiments. Mice in groups of four to six animals were given 220×10^6 trichomonad-hours units of antigen over a 10-day period. Ten days later the animals were challenged with 10^6 organisms inoculated subcutaneously. Four series of immunizations were run. The third and fourth series were carried out at the latter level and also at two, three, four, and six times the level of antigen administered in the first two series. Sham medium inoculated control animals were run with each series. The overall percent-

FIGURE 1. Hematoxylin-stained sections of 5-day-old lesion. A, connective tissue layer; B, medium zone of living trichomonads; C, inner core of caseous material containing degenerate *Trichomonas gallinae;* D, underlying musculature; E, residual agar from initial inoculum.





ages of animals failing to develop lesions was 5 percent in the control group and 50 percent in the immunized group. Twenty-one animals were included in the control group and 37 animals were immunized. Of these, one animal in the non-immunized controls failed to develop a lesion. Nineteen of 37 animals in the immunized group failed to develop a lesion. Little difference was noted between groups immunized with different levels of antigen. Further investigation of the 50 percent protective dose level should be carried out. The results of these experiments are summarized in table 2 including data on lesion size in the treated and control groups.

The equivalent triturated bodies of the trichomonads used to obtain the antigen failed to protect mice in experiments 1 and 2. However, in experiment 3, two of four mice immunized with six times the number of triturated organisms used in previous experiments failed to develop lesions.

The results obtained in this study support the concept that substances secreted or excreted by parasitic organisms may be more important in determining the immune response of the host than those substances which arise as a result of death or destruction of the parasite (Chandler, 1953). The *T. gallinae*-mouse system used in the present study is particularly useful for this type of analysis because the suspending medium used to collect antigens is presumably free of protein material. The results of Thillet and Chandler (1957) demonstrating the production in vitro of antigens capable of inducing protective antibody formation in the rat against *Trypanosoma lewisi* depended upon the presence of non-immune host serum in the incubation mixture. In experiments of the latter type there always existed the possibility that altered host protein, rather than production of parasite substance, might be the antigenic material responsible for the antibodies observed. Stabler (1948b) was able to demonstrate protection of pigeons against virulent *T. gallinae* resulting from acquired immunity using less virulent strains. These results suggested that the failure of Stabler's strain 1 to produce lethal infections or lesions in animals previously infected with the milder strains was due to an acquired humoral immunity. Nonetheless, Stabler did not attempt to demonstrate passive transfer of the protective substance; it is conceivable that the

effects observed were a consequence of some sort of tissue immunity. Our experiments with mice immunized via intraperitoneal inoculation suggest that *T. gallinae* can produce circulating antigens which confer protection against challenge infection. In the experiments carried out in the present study little information was obtained concerning the 50 percent protective dose level of antigen. However, it does appear as though the range of antigen units in which the experiments were carried out was close to a 50 percent level. Although the mouse system is suitable for detection of antigenic substances rendering protection, the sensitivity of the assay method is limited to demonstration of complete protection. Examination of the data in table II shows a wide degree of variation in the size

TABLE II.

Effect of immunization with psysiologically derived substances on the course of subcutaneous infections with T. gallinae *in white mice.*

Experiment	Immunized			Control		
	Mean size*	No. animals Total	No. animals Negative	Mean size*	No. animals Total	No. animals Negative
1**	0	4	4	9.2 (7–10)	5	0
2**	2.8 (0–8)	4	3	10.8 (10–12)	6	0
3***	7.1 (0–13)	16	8	14.0 (11–18)	5	0
4***	0.4 (0–5)	13	4	4.0 (0–6)	4	1
Total			19			1
Mean	2.6	37		9.5	20	
% Negative	51%			5%		

* Diameter of lesion in mm.
** Holtzman mice.
*** Rockland Swiss Webster mice.

of lesion attained in both treated and control groups. For example, in experiment 4 the size of control lesions was significantly smaller than those in previous experiments. It is obvious from this data, that precise quantitation in this system requires large numbers of animals.

The histology of the lesions produced by *T. gallinae* is essentially the same as the "circumscribed subcutaneous abscess" described by Schnitzer et al (1950). In contrast to the similarity of the structural aspects of lesions induced, in our experience the quantitative response, due to a given number of organisms administered, differed markedly from those observed by Schnitzer et al (op. cit.). As shown in table I lesions were not externally visible until the 10th day after inoculation when the number of organisms was reduced from 10^6 to 0.5×10^6. The latter investigators found no difference in the time of appearance of lesions over a thousand fold range of organisms injected. Since the degree of pathology observed in subcutaneous lesions is apparently the same, the differences observed were probably due to the natural resistance of the strain of host utilized for the respective studies. Further evidence of host differences can be adduced from our failure to achieve intraperitoneal infections with the Jones' barn strain (Warren, unpublished data).

Sera of mice which have undergone subcutaneous infection possess agglutinating antibodies (Warren and Chandler, 1958). Similar observations following intramuscular infection of mice with *T. vaginalis* have been reported by Arai (1959). The presence of agglutinating antibodies in sera of infected mice suggests that similar antibodies may occur in the sera of PD antigen inoculated animals. The detection and quantitation of such antibodies would be useful for the identification of the antigenic substance involved in the protective reaction. The results obtained in the present study suggest that the antigen responsible for the protective response could be isolated and readily identified, particularly if this substance is a protein and not a haptene capable of conjugating with native mouse protein. Characterization of this antigen would increase our understanding of immunity to parasitic infection.

SUMMARY

Mice infected subcutaneously with *T. gallinae* have been used as a model to study the role of physiologically derived substances as antigens responsible for immunity in a protozoan infection. In vitro grown organisms were incubated in a protein-free medium for the purpose of collecting antigens. Lyophilized extracts of the incubation mixture were capable of rendering complete protection against challenge infection in 50 percent of the animals tested. Studies have also been carried out on the nature of the infection and the character of the pathology resulting from such infections. The results have been discussed in relation to other studies concerning physiologically derived antigens from protozoa obtained in vitro, and observations on induced immunity to trichomonad infections.

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ANNOUNCEMENT

11TH ANNUAL INSTRUMENT SYMPOSIUM AND RESEARCH EQUIPMENT EXHIBIT

OCTOBER 9 THROUGH 12, 1961

National Institutes of Health, Bethseda 14, Maryland

Instrument Symposium

Committee on Symposium on Recent Developments in Research Methods and Instrumentation

CHAIRMAN	EXECUTIVE SECRETARY
Dr. Julius Sendroy, Jr.	Mr. James B. Davis
Naval Medical Research Institute	National Institutes of Health
Bethesda 14, Maryland	Bethesda 14, Maryland

Primary topics for discussion on the scientific program include: (1) Applied Gas Chromatography; (2) Factors Influencing Interpretation of Spectra; (3) Electron Magnetic Resonance; (4) Thermogravimetric Analysis; (5) Electron Probe Analysis; (6) Application of Physiological Instrumentation to Clinical Problems; and (7) Optical Rotatory Dispersion.

Research Equipment Exhibit

Committee on Manufacturers' Representatives

CHAIRMAN	EXHIBIT MANAGER
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American Instrument Co., Inc.	National Institutes of Health
8030 Georgia Avenue	Bethesda 14, Maryland
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Examples of the latest types of research equipment will be exhibited by instrument manufacturers.

RESEARCH NOTES

FAILURE TO INFECT SPLENECTOMIZED PRIMATES WITH *THEILERIA PARVA*.

Following success in infecting splenectomized primates with bovine piroplasmosis such as *Babesia divergens* reported by Garnham and Bray (1959, J. Prot. **6**: 352–355), it was decided to make a similar attempt using *Theileria parva*, the causative organism of East Coast Fever.

Some 70 *Rhipicephalus appendiculatus* 70 percent infected with *T. parva* were most kindly sent to the Liberian Institute by Dr. D. W. Brocklesby of the East African Veterinary Research Organization. Of these 20 were applied to chimpanzee No. 25 which had been splenectomized 2 years previously, and another 20 were applied to chimpanzee No. 59 which had been splenectomized just prior to the time of application. The ticks were left on for 8 days. Fourteen attached and partially fed on No. 25 and one was attached and engorged on No. 59. All fed ticks were then removed, ground up in physiological saline, and inoculated intraperitoneally into both animals; No. 59 received two-thirds of the suspension. Both chimpanzees failed to show *T. parva* in the blood during the following 3 months.

Another 20 ticks were applied to a *Cercocebus torquatus atys* (sooty mangabey) which had been splenectomized just prior to the application. These were left on the monkey for 14 days during which ten engorged. The monkey died 30 days after application and never showed piroplasms in the blood.

At the same time additional *R. appendiculatus* infected with *T. parva* were sent by Dr. Brocklesby to the London School of Hygiene and Tropical Medicine. Of these 15 and 12 were applied, respectively, to two *Macaca mulatta* (rhesus monkeys) which had been splenectomized 28 and 24 months previously. One tick engorged on the first monkey and seven on the second. In the latter case, the ticks were ground up in physiological saline and the suspension was inoculated intraperitoneally into the same animal. A lymph gland was removed from this animal 4 weeks after application, sectioned, and examined for Koch's blue bodies. No *T. parva* was found in the gland sections and the blood of both animals showed no piroplasms during repeated examinations during the following weeks.

We wish to record our gratitude to Dr. D. W. Brocklesby for making infected ticks available to us. (Supported in part by grant E2178, National Institutes of Health, U.S.P.H.S.)—
R. S. Bray, *Liberian Institute of the American Foundation for Tropical Medicine, Inc., Harbel, Liberia,* and P. C. C. Garnham, *London School of Hygiene and Tropical Medicine, Keppel St., London, W.C.1, U.K.*

ADDITIONAL RECORDS OF HUMAN CASES OF *DIPYLIDIUM CANINUM* INFECTIONS.

Recent interest in the reporting of human infections with *Dipylidium caninum* in the United States will undoubtedly increase the surprisingly low number of 14 thus far appearing in the literature. Hutchinson et al (1959, Am. J. Trop. Med. Hyg. **8**: 603), Moore and Connell (1960, Am. J. Trop. Med. Hyg. **9**: 604–605), and Olsen and Box (1960, Texas State J. Med. **56**: 740) have recently reviewed the literature, and each has reported new cases.

The present author has seen this parasite from three children in the Memphis, Tennessee, area in the past 5 years. In two cases, no further record is available. He is indebted to Dr. Barton C. Etter for the third case. This was a 5-year-old white girl living in this city who had a pet cat which she frequently fondled and played with. This close association with her pet and the fact that her mother noticed "exactly the same kind of worms" crawling over the cat's feces suggests the animal may have been the reservoir host. The cat had been treated before it's stools were studied, however, and no parasites were found. Also, on occasion dogs did visit this child's home. On the basis of chance it would appear that the cat was most probably involved; in this respect the case resembles that of Olsen and Box. The child received quinacrine hydrochloride and was apparently cured of the infection. To the author's knowledge this brings to a total of 17, the number of reported human cases of *Dipylidium caninum* infections in the United States.—Robert C. Rendtorff, *Section on Epidemiology, Division of Preventive Medicine, The University of Tennessee, College of Medicine, Memphis.*

GROWTH CHARACTERISTICS OF CLONES OF *TRICHOMONAS VAGINALIS* IN SOLID MEDIUM*

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Trichomonas vaginalis has been cultivated in many different types of liquid medium. C.P.L.M. media (Johnson and Trussell, 1943), simplified trypticase medium (Kuperferberg et al, 1948) and a medium devised by Diamond (1957) probably have had the widest use.

There have been few reports of growth of *T. vaginalis* in solid media. Wirtshafter (1954) described a culture overlay method of maintaining *T. vaginalis* on slants for 20 or more days. Asami et al (1955) described methods of growing the organism on media in petri plates and also in stab cultures. Filadora and Orsi (1958) described methods of growing the organism on the surface of media or by a pour plate technique. In all of the above reports either isolated colonies were not reported or the method was not satisfactory for serial transfer of organisms.

The following report describes a convenient pour plate technique for obtaining isolated clones of *T. vaginalis* which may be serially transferred. Also, certain growth characteristics of *T. vaginalis* in this medium are described.

MATERIALS AND METHODS

Stock cultures of *T. vaginalis* were maintained in liquid medium described by Diamond (1957). The medium utilized for the pour plate technique was identical to the maintenance medium except ionagar, product of Consolidated Laboratories, Chicago, Illionis, in a concentration of 0.45 percent, was substituted for agar. To prepare 100 ml of pour plate medium, the following procedures were carried out.

1. 2.0 gm of trypticase (BBL), 1.0 g of yeast extract, 0.5 g of maltose, 0.1 g of cysteine HCl, and 20 mg of ascorbic acid were added to 75 ml of water, and the pH adjusted to 6.0. Then, 0.45 gm of ionagar was added and heated to 100 C until ionagar was in solution.

2. Medium was dispensed into tubes in 7.5 ml amounts before agar solidified and then was autoclaved at 121 C for 10 minutes.

3. The tubes of medium were cooled to 43 to 45 C in a water bath and 1.0 ml of serum and 1.0 ml of a penicillin, streptomycin, mycostatin mixture were added to each tube. Final concentration of antibiotics per ml of medium was 100 units penicillin, 100 micrograms streptomycin, and 25 micrograms mycostatin.

4. The inoculum, contained in 0.5 ml of regular Diamond medium, was pipetted into a sterile petri dish and a tube of medium containing serum and antibiotics was poured on top of inoculum, mixed and allowed to harden. A petri dish blotter was inserted to control excess moisture.

5. The petri dish was placed in Brewer jar and the air replaced with illuminating gas, using standard Brewer jar techniques.

start w.m.

Colony Growth, Description

Maroscopically growth was not obvious until the third day after inoculation. On the third day the colonies were 0.25 mm or smaller, and difficult to discern.

Figures 1 through 4 depict colony growth in petri plate medium from the fourth through the seventh day after inoculation. Isolated colonies were readily visible by the fourth day (fig. 1). By the fifth day colonies had reached their maximum size (fig. 2). Colony size ranged from 0.2 to 2.0 mm with most colonies measuring between 0.5 and 1.0 mm in diameter. Typical colonies were subsurface in location, smooth, opaque circular, and dull cream in color. As shown in figures 3 and 4, the medium tended to become cloudy by the sixth and seventh day after inoculation. Also, colonies became slightly dark in the center after the seventh day. These studies indicated that macroscopic colony counts were best done on the fifth day after inoculation.

Microscopic studies were carried out at 45 magnifications. Although morphological detail was not clear at this magnification (as long as the discrete colony was left intact), typical trichomonad movement could be observed if the medium was ruptured (freeing the individual organisms). Studies of colony growth over a period of days at higher magnification were not

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practical because the objective lens disrupted the surface of the medium when subsurface colonies were brought into focus.

Figures 5 through 8 depict characteristic microscopic colony growth from the second through the fifth day after inoculation. On the second day the colony was an irregular, amorphous mass with a diameter of about 130 microns (fig. 5). On the third day the colony reached a diameter of about 220 microns and assumed a more or less spherical shape which it retained the remainder of its development (fig. 6). By the fourth day the diameter of the colony had increased to 440 microns (fig. 7), and on the fifth day the colony reached its maximum size of approximately 720 microns (fig. 8).

Figures 9 and 10 depict a different growth pattern of three colonies seen in the same field. Relative growth may be compared with a spherical artifact seen in these figures. On the third day of growth, three colonies were noted ranging in size from 170 to 400 microns (fig. 9). By the sixth day, colony diameters ranged from 420 to 700 microns and a migration of organisms into the microscopic field was obvious as evidenced by the clearly discernible individual organisms (fig. 10). This type of migration was apparent on the fourth day after inoculation and occurred at the bottom of the medium between the medium and the petri plate. Apparently organisms in this position were able to spread either by locomotion or by pressure of dividing organisms pushing others ahead.

Colonies located in the agar remained discrete and never spread whereas colonies originating either on the bottom of the medium or more rarely on the surface, spread haphazardly as seen in figure 10, or as a concentric ring or "halo" surrounding the centrally located colony. Figure 11 shows a petri plate with colonies that exhibited concentric spread of organisms from the centrally located colonies. This type of spreading increased the colony diameter an additional 2 to 9 millimeters.

Effect of Varying Agar Concentrations

Plates of Diamond medium were prepared with varying concentrations of ionagar and agar agar. Each plate received an inoculum of 150 organisms. The experiment was done in duplicate and repeated twice. As shown on table I, plates containing 0.35 percent ionagar had an average of 140 colonies; those plates containing 0.45 percent ionagar averaged 118 colonies while those plates containing 0.55 percent ionagar averaged 112 colonies. Higher concentrations of ionagar did not allow appreciable colony development. Also, it was noted that colony diameters decreased as the concentration of ionagar increased. Although 0.35 percent ionagar allowed more colonies to develop, there was a tendency for the agar surface to crumple or collapse if plates were tilted. For this reason 0.45 percent concentration of ionagar was preferred.

TABLE I. *Effect of varying agar concentrations on colony development of* Trichomonas vaginalis.

Concentration and type agar used	Average* number of colonies developing
0.35 pct. ionagar	140
0.45 pct. ionagar	118
0.55 pct. ionagar	112
0.65 pct. ionagar	Negligible
0.75 pct. ionagar	Negligible
0.5 pct. agar	125
0.7 pct. agar	Negligible

* Each plate received inoculum of 150 organisms. Experiment done duplicates and repeated twice.

Colony development was adequate in medium containing 0.5 percent agar agar as shown in table I. No growth occurred in medium containing 0.7 percent agar while medium containing 0.3 percent concentration of agar did not solidify. However, agar agar caused an opaque medium, and made colonies harder to enumerate. Ionagar, in contrast, created a clear medium in which colonies were much easier to detect.

Effect of Air and Temperature

Inoculated plates were exposed to air for 6 and 18 hours at 25 C and 37 C before subsequent anaerobic incubation. Other inoculated plates, under anerobic conditions, were subjected to 25 C for 24 and 48 hours before subsequent incubation at 37 C. Inoculated control plates were maintained anaerobically and aerobically at 37 C throughout the experiment. The experiment was done in duplicate and repeated twice, each plate receiving an inoculum of 150 organisms. Colonies on all plates were counted during the seventh day after inoculation.

As shown in table II, plates incubated anaerobically at 37 C had an average of 141 colonies. Those plates kept anaerobically at 25 C for 24 and 48 hours had an average of 68 and 18 colonies, indicating the adverse effect of this treatment. Plates exposed to the air for 6 hours at 37 C and 25 C had an average of 115 and 124 colonies respectively. While plates exposed to air 18 hours at these two temperatures averaged

22 and 75 colonies, indicating the adverse effect of prolonged exposure to air. No colonies ever developed in plates incubated aerobically at 37 C throughout the experiment.

Survival Time in Pour Plate Medium

Six plates were inoculated with 150 organisms and incubated anaerobically at 37 C for five days. On the fifth day the plates were divided into three equal groups and further incubated anaerobically at 37 C, 25 C, or 5 C. Viability checks were made daily by excising colonies and inoculating into liquid medium. Results indicated that organisms on plates incubated anerobically at 37 C throughout were able to survive seven days. Organisms on plates placed at 25 C on the fifth day were able to survive 9 days. Organisms on plates placed at 5 C on the fifth day did not survive past the sixth day.

Although all the results reported on here used illuminating gas as the anaerobic atmosphere, subsequent work showed that an atmosphere of nitrogen was slightly superior to illuminating gas. Organisms in an atmosphere of nitrogen survived as long as 11 days (after being incubated at 25 C from the fifth day on). Also, an additional advantage is afforded as a methylene blue indicator system can be included in the Brewer jar to indicate whether the system is anaerobic.

TABLE II. *Effects of aerobic and anaerobic conditions at 25 C and 37 C in subsequent colony development of* Trichomonas vaginalis.

Environment	Temperature	Length of exposure	Average* number colonies developing
Anaerobic	37 C	Until termination	141
Anaerobic	25 C	24 hours	68
Anaerobic	25 C	48 hours	18
Aerobic	37 C	6 hours	115
Aerobic	37 C	18 hours	22
Aerobic	25 C	6 hours	124
Aerobic	25 C	18 hours	75
Aerobic	37 C	Until termination	0

* Each plate received inoculum of 150 organisms. Experiment done in duplicates and repeated twice.

Serial Transfer of Organisms

Transfer of organisms from a single colony to new pour plates was readily effected. Using aseptic technique a colony was excised, macerated in a sterile mortar and pestle using a small amount of liquid medium. Hemocytometer counts, to determine the number of organisms present were performed. From this information appropriate dilutions were made to arrive at the desired inoculum size. This procedure has been repeated many times. Quanitative counts of the number of organisms in colonies revealed that 5-day-old colonies with diameters between 1.0 and 1.5 mm contained roughly 100 organisms per micron colony diameter. Thus, a 1.0 mm diameter colony usually contained approximately 100,000 organisms.

DISCUSSION

The pour plate technique described in this report provides a simple method for obtaining isolated colonies of *T. vaginalis*. Although colonies were visible to the naked eye by the third day after inoculation, colony counts were best done on the fifth day as by this time maximum colony size was attained. Colonies were easily transferred from plate to plate as described above. However, if clone selection was desired, care had to be taken that organisms from other areas of the plate had not migrated and mingled with the clone to be transferred. This type of migration was observed to take place at the bottom of the medium or less frequently at the surface, but never within the agar medium.

There were certain physical limitations of the technique that had to be taken into consideration if successful results were to be obtained. The concentration of ionagar had to be within a 0.35 to 0.55 percent range. Lower concentrations did not solidify the medium and higher concentrations would not allow growth. Also, exposure of inoculated plates to atmospheric conditions and/or 25 C for periods as short as 18 hours proved decidely detrimental. Evidently, even though the organisms are in a medium with a low oxidation-reduction potential, it is necessary to incubate the plates anaerobically rather soon after inoculation to prevent organism deterioration. This may be due to the fact that the 10 ml of medium in a standard petri plate does not provide deep enough medium to protect the organisms from oxygen. It is surprising that 25 C temperatures prove detrimental so quickly. Organisms survive in liquid maintenance medium at this temperature for several days. However, the number of organisms in the liquid medium is invariably much greater (from 10,000 to 1 million per ml) and the presumed superior survival ability in liquid medium may be due only to the larger

inoculum providing sufficient number of organisms to survive and initiate growth once optimum temperatures are provided.

The technique described in this report for obtaining isolated clones of *T. vaginalis* will make possible studies on this organism that have not been possible before. Clone selection will make more valid studies on strains of *T. vaginalis*. Also, chances of detecting organism variation, either natural or after experimental stress, will be greatly enhanced. In the past the effects of physical and chemical agents on this protozoon could be measured only in terms of immobilization of the organism or total kill, i.e., inability to subculture the organism. With the plate technique an accurate quantitative measurement of the effect of physical and chemical agents may be obtained. Also, the plate technique may make possible studies on nutritional requirements and drug sensitivity in a manner not previously available. Quite likely the plate technique described here could, with suitable modifications, be utilized in studies of other protozoa.

SUMMARY

A plate technique was described for obtaining clones of *T. vaginalis*. Under anaerobic conditions organisms developed into clonies ranging from 0.2 to 2.0 mm in size in about 5 days. Macroscopic and microscopic colony growth over a period of days were described. Certain limitations of the procedure were investigated and clarified. Clone growth occurred in medium containing ionagar within the range of 0.35 and 0.55 percent. Prolonged exposure of organisms to oxygen and/or 25 C proved detrimental to clone development.

Potential uses of the procedure were discussed.

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EXPLANATION OF PLATE I

Figures 1, 2, 3, and 4 are photographs of the growth sequence of *T. vaginalis* colonies on a petri plate from the fourth to the seventh day after inoculation.

FIGURE 1. Colony growth, fourth day.
FIGURE 2. Colony growth, fifth day.
FIGURE 3. Colony growth, sixth day.
FIGURE 4. Colony growth, seventh day.

Figures 5, 6, 7, and 8 are photomicrographs (45 magnifications) of the growth sequence of a colony of *T. vaginalis*.

FIGURE 5. Colony growth, second day.
FIGURE 6. Colony growth, third day.
FIGURE 7. Colony growth, fourth day.
FIGURE 8. Colony growth, fifth day.

EXPLANATION OF PLATE II

Figures 9 and 10 are photomicrographs (45 magnifications) of *T. vaginalis* colonies on third and sixth days after inoculation.

FIGURE 9. *T. vaginalis* colonies, third day.

FIGURE 10. *T. vaginalis* colonies, sixth day. Note spread of organisms throughout the medium.

FIGURE 11. *T. vaginalis* colonies, 7 days old, exhibiting concentric rings of growth from parent colonies.

PLATE I

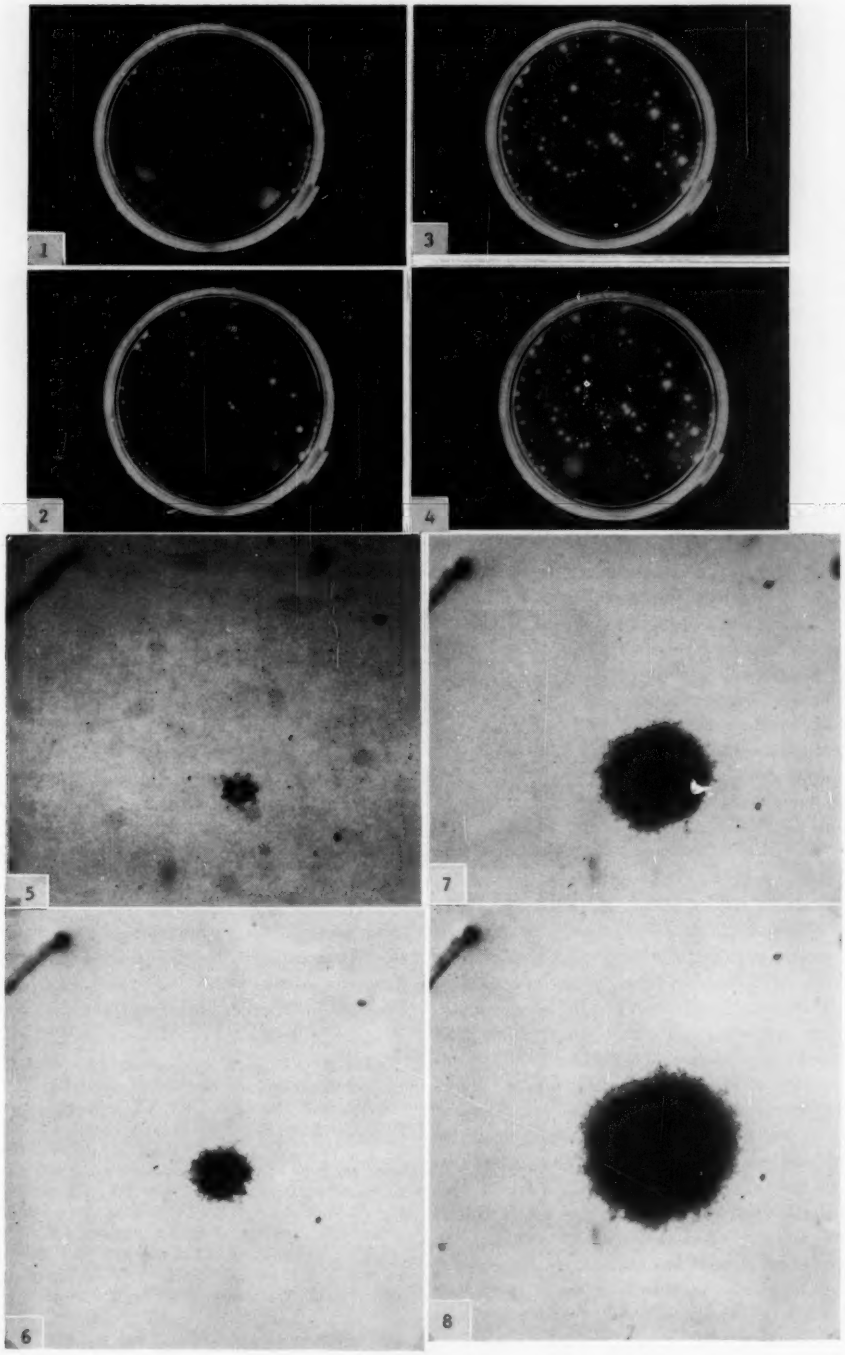
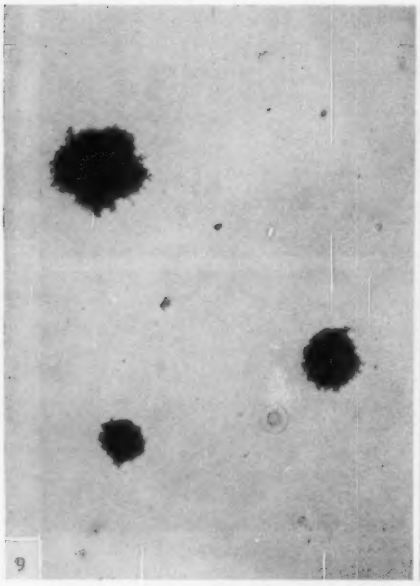
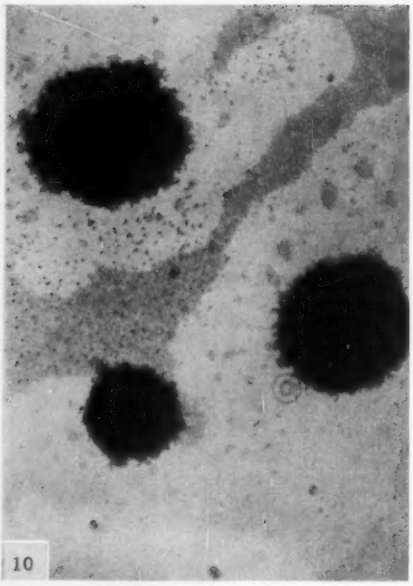
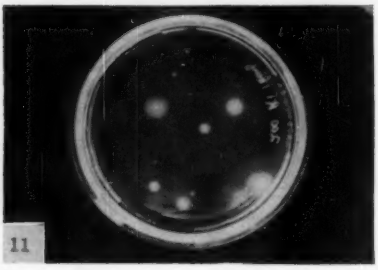


PLATE II







COMPARATIVE PATHOGENICITY OF *TRICHOMONAS VAGINALIS* AND *TRICHOMONAS GALLINAE* TO MICE. I. GROSS PATHOLOGY, QUANTITATIVE EVALUATION OF VIRULENCE, AND SOME FACTORS AFFECTING PATHOGENICITY* **

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For over a quarter of a century mice have served as experimental hosts of several trichomonad species. One of the earliest reports concerning intraperitoneal, intrathoracic, intramuscular, and subcutaneous inoculations of bacteria-free *Trichomonas gallinae* (Rivolta) was published by Bos (1934). Wagner and Hees (1935) and Wagner (1938) confirmed Bos' reports in reference to intraperitoneal inoculations, but it is not absolutely clear if they employed axenic cultures of parasites in their experiments. Other records of inoculations of mice with *T. gallinae* were those of Wittfogel (1935) and Miessner and Hansen (1936). Morgan (1942) succeeded in infecting mice with bacteria free *Tritrichomonas foetus* (Riedmüller); also, he reviewed previous reports dealing with inoculation of this species into mice. Schnitzer et al (1950) described the gross pathology and some histopathology of intraperitoneal, intramuscular, and subcutaneous lesions produced by inoculations of single axenic strains of *Trichomonas vaginalis* Donné, *T. gallinae* and *T. foetus*. Of the three species only the strain of *T. foetus* produced fatal infections when inoculated intraperitoneally. The authors measured the intramuscular abscesses only, but even in reference to these lesions no statistical comparison was attempted. In conclusion the investigators stated: ". . . trichomonads of avian origin produced purulent lesions of specific character in mice . . . identical infections were obtained with one strain each of *T. vaginalis* and *T. foetus*. The latter organism appeared as a rule more virulent than *T. gallinae* which in turn impressed us as being more pathogenic for mice than *T. vaginalis*." In view of the fact that the authors worked with a single strain of each species, their conclusions might have been justified in reference to these particular strains but, as our studies indicate, did not necessarily reflect the relative pathogenicity of all strains of these species. In the same paper, they reported the employment of the subcutaneous route of inoculation as the most suitable "for the evaluation of therapeutic effects exerted by topical application of chemotherapeutic agents."

Since 1950 there have been numerous reports dealing with comparative pathogenicity and immunology of trichomonad species. Mice served as experimental hosts in many of these studies. A survey of the germane literature reveals that much work on the effects of trichomonads, especially *T. vaginalis*, on mice is being done in Japan and the Soviet Union and some in this country.

Among the Japanese workers, Hamada (1953) published a report on the effects of intraperitoneal inoculations of *T. vaginalis* into mice. The general pathology of the lesions, including the involvement of the liver did not differ materially from those reported by Schnitzer et al (1950). The same was true of the pathological changes described by Yamagata (1954) whose study included also *T. foetus*. In his extensive report, Iwai (1957) presented an excellent discussion of previous research done in Japan during the last decade on pathogenicity of trichomonads to mice. The report included also much interesting new material concerning the effects of intraperitoneal, intrapleural, intramuscular, and subcutaneous inoculations of several strains of *T. vaginalis* on laboratory mice. Unfortunately, Iwai's shortest

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series of experiments was that involving the subcutaneous route, and all inoculations in this series involved a single strain of the genital trichomonad. However, he was able to detect differences in pathogenicity among three strains by titrating them with the aid of intraperitoneal inoculations. The injection of equal numbers of organisms belonging to three separate strains gave different results in terms of the percentage of animals which developed the typical abscesses and ascitis. It will be noted, however, that the samples were relatively small; in most instances there were 10, in one as few as 5, and only in one case as many as 16 treated animals. The pathology of the lesions was again quite similar to that reported by all previous investigators. The strains of *T. vaginalis* employed by Schnitzer et al (1950), the Japanese, and the Russian worker Vershinskii (1958) failed to kill the hosts, although they produced typical lesions, including hepatic abscesses. On the other hand, the investigations of Bogovsky and Teras (1958) in Russia and Reardon and Jacobs (1958) in this country clearly demonstrated that there were some strains, for example C_1 of Jacobs, which do cause the death of experimental animals. Thus, these strains appear to be as pathogenic to mice as those of *T. foetus*. While Reardon and Jacobs merely stated that their other strain, R, failed to kill mice. Bogovsky and Teras differentiated the pathogenicity of several strains on the basis of gross and histopathological characteristics of the lesions, the time necessary for their development, and the length of time needed to kill the mice.

When the investigation reported in this paper was begun, we searched for some simple means of strictly quantitative evaluation of the pathogenicity of strains of *T. vaginalis* and *T. gallinae*. We were aware also of the fact that despite many statements in the literature, for example Trussell (1947) or Holtroff (1957), the pathogenicity of the genital trichomonad of man has not been unequivocally accepted by all parasitologists or clinicians. On the other hand, no one doubts that some strains of *T. gallinae*, for example Stabler's Jones' Barn strain, which kill their natural hosts within a week, are true pathogens. Thus a comparative study of the effects of strains of the avian and human species in a single type of susceptible host would, in our opinion, provide further proof of pathogenicity of the human parasite, especially if there were a close correlation between the degree of virulence which the strains exhibit in mice and in the natural hosts. As will become apparent from the discussion that follows, among the strains of *T. gallinae* excellent correlations exist between the level of pathogenicity of these strains to mice and to pigeons. There are also many indications that such a correlation may be found among the strains of *T. vaginalis*. Bogovsky and Teras (1958) stated: "A definite connection between the clinical condition . . . in examined patients and the pathogenicity of *T. vaginalis* in white mice has been established." This statement agrees with our preliminary findings. However, a series of tests in collaboration with Dr. John K. Frost, the Johns Hopkins School of Medicine, is now in progress; that study will involve a large number of strains freshly isolated from patients showing a variety of clinical symptoms connected with trichomonad infection. After accumulation and statistical analysis of the data derived from these experiments, we should be able to establish unequivocally if the correlation between the degree of pathogenicity of the strains of the human genital trichomonad to the natural hosts and to mice is as high as that found for the strains of *T. gallinae*.

It was felt that an effective test for virulence should be relatively simple, and even more important, should yield quantitative data suitable for statistical analysis. The extent of intraperitoneal lesions or of the degree of the pathological changes is certainly very difficult to evaluate quantitatively. Since many strains of *T. vaginalis* or of *T. gallinae* fail to kill mice, even if a very large number of parasites is inoculated, evaluation of the virulence of the strains in terms of a lethal dose is impractical. Titration of the various strains on mice [such as employed by Iwai, 1957 (see above)], by calculating the percentage of animals showing pathological changes after inoculation of identical numbers of flagellates would necessitate a very large number of treatments and a very careful examination for even the slightest indication of such changes, if the "all-or-none" rule were to be applied. In view of the foregoing the intraperitoneal route of inoculation, which has been widely employed by previous investigators, was rejected for the purpose of our tests. The method used by Schnitzer et al (1950), according to which the intramuscular

lesions were excised and measured, seems to be very acceptable from the quantitative point of view. At the same time, however, it is quite time-consuming and would not be suitable for more than one reading. The latter objection is, of course, equally valid in reference to all the methods of evaluation which involve intraperitoneal injections. Thus it became evident that subcutaneous lesions would suit our purpose best, and some means had to be devised whereby such lesions could be measured repeatedly with a sufficient degree of accuracy. In order to assure the availability of large samples, a quantity of parasites was found which, under standard conditions, caused lesions in nearly 100 percent of inoculations with even the mildest strains. It will be noted, however, that in some series of experiments, particularly those in which the effect of environmental factors upon pathogenicity was tested, the percentage of positive versus negative tests was evaluated also.

No one can doubt that, aside from the genetic makeup of the parasite (see Honigberg and Read, 1960), various factors involving the general host physiology as well as those present in or acting at the immediate site of the parasite must play a role in the final expression of its virulence. However, as suggested by Stabler and Engley (1946) on the basis of experimental infections of clean pigeons, it appears that environmental conditions, for instance the kinds of bacteria found in the natural hosts, do not play an important role in enhancing pathogenicity of virulent strains of *T. gallinae*. Trussell (1947), having reviewed the data obtained by himself and others from many series of experimental inoculations of women, concluded that ". . . *T. vaginalis* can cause vaginitis regardless of the bacterial flora initially present;" however, referring to the work of Hesseltine, Wolters, and Campbell (1942), he also stated, "These authors thus were able to confirm the observation that clinical vaginal trichomoniasis can be produced by inoculation of the human vagina with a bacteria-free culture of *T. vaginalis*. In addition, they clarified and properly emphasized the relation of abnormal vaginal flora to susceptibility." There can be no question that, as demonstrated by Stabler (1948), strains of *T. gallinae* that exhibit different degrees of virulence to clean, experimentally infected pigeons do exist. In the light of the works reviewed by Trussell (1947) and on the basis of reports by other American (Reardon and Jacobs, 1958), Japanese (Iwai, 1957), and Russian (Bogovsky and Teras, 1958), investigators it seems that strain differences in virulence to the natural hosts may be found also in the human genital trichomonad.

Mice, which are quite susceptible to infection with both of these trichomonad species, can serve conveniently for evaulation of relative pathogenicity of both *T. gallinae* and *T. vaginalis*. In the case of the latter species it would certainly be easier to make such evaluations on mice than on volunteer women. Furthermore, if one assumes, perhaps quite legitimately, that the nearly homozygous healthy mice, all of the same age, provide a relatively uniform environment, one could test the influence of some of the environmental factors upon the pathogenicity of a given strain. Such tests were performed and are described below.

The present paper deals with most aspects of pathogenicity of various strains of *T. vaginalis* and *T. gallinae* to mice and represents a complete report of the investigations started in 1956 and summarized briefly in previous abstracts by Honigberg and Braunthal (1957) and Honigberg (1959); however, a study of the histopathology caused by the two species in mice will be published in a separate article.

MATERIALS AND METHODS

A. Strains of Parasites

The following strains of *Trichomonas vaginalis* Donné and *T. gallinae* (Rivolta) were employed in the present investigation:

1. *Trichomonas vaginalis*
   a. TV 1, Strain 1, isolated by Dr. Ray Trussell in 1939, has been maintained in culture by Dr. A. B. Kupferberg, who sent it to us in 1955.
   b. TV 3, Strain 3, isolated by Dr. Garth Johnson in 1945, has been maintained in culture by Dr. Kupferberg, who sent it to us in 1955.
   c. TVC, Cavanagh strain, was isolated in our laboratory in April 1956 from a sample of discharge obtained by Dr. James E. Cavanagh from a severe case of vaginitis. The strain has been maintained by us since the time of its isolation.
   d. TVC_1, C_1 strain, isolated by Dr. Leon Jacobs in September 1956 from a very acute case of vaginitis, was sent to us by this investigator in March 1960.
2. *Trichomonas gallinae*

a. JB, Jones' Barn strain, of Dr. Robert Stabler was isolated in culture in our laboratory in late August 1956 from the liver of an experimentally infected pigeon. The bird was infected from pigeon #275 in Stabler's consecutive infection series. The strain had been maintained by us until April 1958, at which time it was contaminated with an unidentified coccus which changed the physiology of the protozoan. The bacterial strain was eliminated from the culture with the aid of antibiotics; however, because the physiology of the trichomonad appeared to be permanently affected, the culture was discarded.

The Jones' Barn strain was isolated again in July 1958 from the liver of an experimentally infected pigeon sent to us by Dr. Stabler. This time axenic cultures were isolated both with and without the aid of antibiotics. The strain isolated without antibiotics is the only one employed in the present investigation.

b. YG, Lahore strain, of Stabler was isolated in culture in our laboratory in late August 1956, from the mouth of an experimentally infected pigeon sent to us by Dr. Stabler. It has been maintained by us since the time of its isolation.

c. TG, Kupferberg strain, isolated by Dr. A. B. Kupferberg in 1947 from the crop of a pigeon, has been maintained by him in culture since that time and was sent to us in 1955.

TGNA represents TG strain which has been maintained on STS medium (see below) without agar since March 1955.

d. AG, Amherst strain, was isolated in our laboratory in December 1956 from the mouth and esophagus of a pigeon. The strain is being maintained in our laboratory.

It should be noted that strains TVC (4/10/56) and TVC_1 (9/11/56) of *T. vaginalis* and strains JB (8/29/56), YG (8/24/56), and AG (12/24/56) of *T. gallinae* were isolated in culture close in time to one another; strains TVC_1, JB, and YG only a few days apart. This fact has proved to be of importance in our studies (see below).

The antibiotics employed routinely in the isolation of the axenic cultures of strains of both species of parasites were crystalline penicillin G (5 to 10×10^3 units per ml) and dihydrostreptomycin (1 to 5×10^3 micrograms per ml).

B. Culture Media

Several media have been used for the maintenance of and experiments with the various strains. However, BBL Fluid Thioglycollate (FT) with 1 percent normal horse serum (FTS 1) for the strains of *T. gallinae* and with 5 percent normal horse serum (FTS 5) for those of *T. vaginalis* were found to be very satisfactory and have been employed routinely for the maintenance of all the base strains. Since, as is well known, avian and human parasites have different pH requirements (Cf. Johnson and Trussell, 1943; Stabler, 1954), the media were adjusted to pH 7.0–7.2 for the former and to pH 5.8–6.0 for the latter.

Except where indicated otherwise, all standard experiments for comparison of pathogenicity of the several strains of both species were performed on FTS 5. In as many series of experiments as were possible, the same batches of FT and of serum were used.

In various experiments other than those mentioned above, simplified trypticase medium with 5 percent normal horse serum (STS) of Kupferberg, Johnson, and Sprince (1948) or the same medium modified by the omission of agar was employed. STS was also used in the series of experiments in which substances other than agar (viz, methyl cellulose, mucin, and gelatin) were substituted at concentrations which gave a viscosity equal to that of STS with agar. It was established with the aid of Oswald tubes that at 37 C the viscosity of the medium obtained with 100 mg percent agar was equalled by the addition of 75 mg percent methyl cellulose, 300 mg percent Wilson's granular mucin, type 1701 W, and 800 mg percent pure bacto gelatin (Difco).

CPLM (cysteine-peptone-liver-maltose) medium of Johnson and Trussell (1943), with five percent normal horse serum was often employed for original isolation of strains of both species, and is still being used, along with FTS 5, for the maintenance of the slow-growing TVC_1 strain of Jacobs. The same medium modified by the omission of agar was utilized in the final series of experiments for testing the progressive attenuation of JB strain grown in the absence of agar.

C. Counting of Trichomonads and Preparation of Growth Curves

All counts of flagellates given in this paper were made with the aid of a hemocytometer. In all instances the counts were made at least in duplicate. In our experience, while no fewer than 12 counts of a culture must be made to insure absolutely dependable and reproducible results, duplicate counts are quite satisfactory for most purposes.

With the exception of TVC_1, the growth curve of which was made at a considerably later date, the curves of all the other strains of both species represented in figure 1 were based on cultures grown on the same batch of FT to which serum from the same batch was added. STS with agar, without agar, and with other substances (see above), used in growing JB strain for the purpose of preparation of the curves represented in figure 5, contained trypticase from a single bottle; the serum came from the same batch. Before being employed for growth curves on such experimental media, the parasites were transferred 3 times on these media. In all instances the original inocula were taken from 48-hour cultures. The counts were made at 4-hour intervals for a total of 60 hours. The portions of the curves representing the phases of logarithmic growth were fitted with the aid of the "least mean square" method, and the generation times calculated according to the standard formula,

$$G = \frac{t \log 2}{\log b - \log a}.$$

D. Estimate of Multiplication Rates of Trichomonads in Subcutaneous Lesions

The growth rate of the parasites in the lesions was estimated according to the following method: (a) The contents of several 2-, 3-, 5-, 7-, 9-, 11-, and 14-day lesions of each strain were suspended separately in 5 ml of 0.154 M NaCl; (b) 0.02 ml aliquots were placed on slides and covered with No. 1 round 22 mm coverslips; (c) The number of trichomonads was estimated in 5 to 10 fields of the microscope (real diameter of field 1.5 mm).

The estimates so obtained are not accurate enough to be treated statistically. They merely represent trends exhibited by the several strains.

E. The Mice

A total of over three thousand 6- to 8-week-old C57 B1/6 mice, purchased from Jackson Memorial Laboratory at Bar Harbor, Maine were used in the experiments. This particular strain of mice was selected because of its sturdiness and resistance to spontaneous tumors. In the earlier experiments both sexes were employed in equal proportions. Since, however, no statistically significant differences could be detected in the response of the sexes to the parasites, male mice which are somewhat cheaper and more readily available were utilized in many of the later series of inoculations.

F. Inoculation of Parasites, Measurements of Lesions, and Calculation of Volumes of the Abscesses

1. All experiments involved subcutaneous inoculations of the parasites into the mid-region of both flanks of the mice. No fewer than 15 (usually more) inoculations of a single parasite strain were made in each experiment. The mice were shaved and the flanks treated with merthiolate prior to inoculation. All the inocula were 0.5 ml and contained 8 to 9×10^6 trichomonads. The selection of this particular number of organisms was based on our findings (through titration of several strains of both trichomonad species) that this size inocula of even the mildest strains, injected in agar-containing media reasonably soon after their isolation in culture, resulted in producing 100 percent lesions.

In over 90 percent of experiments the lesions were spot-checked on liquid and solid media for the presence of microorganisms other than trichomonads. In nearly all instances the only organism that could be isolated was *Trichomonas*. If, as happened on two occasions, even one lesion was found to contain foreign microorganisms, the entire experiment was discarded.

All experiments were controlled by inoculations of 0.5 ml of the same sterile media which served for the cultivation of the parasites employed in the particular experiment. If the edema caused by the injection of the sterile media failed to disappear prior to 48 hours, the mean volume of the "control lesions" was subtracted from that of the experimental abscesses for as many days as the former lesions persisted.

2. In many experiments the measurements were made daily between the 2d and 7th days following inoculation and every 48 hours from the 9th to the 14th day. No measurements were made during the first day because often 24 to 48 hours were necessary for the absorption of the edema caused by the injection of the relatively large volume of liquid.

It was established in the course of this investigation that all the necessary information concerning the differences among the strains could be derived for *T. gallinae* from measurements of the 5th- or 6th day lesions and for *T. vaginalis* from the measurements of the 6th- or 7th-day abscesses. This was equally true for the data obtained on the 12th to 14th day following inoculation. Since the older lesions were always larger, the experimental error in the measurements was correspondingly smaller. In view of this, many of the experiments performed during the later phases of this project were measured on the 5th, 6th, or 7th and 12th or 14th day only. Actually, the latter sets of measurements would have been quite sufficient; however, a certain percentage of abscesses caused by the more virulent strains open and start draining by the end of the first or at the beginning of the second week following inoculation; in some instances only relatively few intact lesions are found by the end of the second week. Hence, experiments involving such strains had to be read earlier if a sufficient number of individual measurements were to be secured to justify statistical analysis of the data.

3. The length and width of the protruding part of the lesion could be conveniently measured with the aid of calipers, and the height by means of a metric rule. It is often helpful to extend a piece of thread or of paper between the top surface of the lesion and the rule. With some experience such measurements can be reproduced with considerable accuracy. On numerous occasions three persons independently obtained closely comparable results. The most important factors in obtaining reproducible results by this method are experience, accuracy, and patience. In our experience, one person must hold the mouse still, without distorting its shape, while the other does the measuring.

4. One of the questions which faced us was the comparison between the actual and the measured (by the method described above) volume of the protruding part of the lesion. "Jelltrate" (The L. D. Caulk Company), elastic impression material used by orthodontists, proved to be admirably suited for making permanent impressions of the lesions. It was assumed that the volume of water necessary to fill an impression would represent the actual volume of the protruding part of the lesion. The actual volume, thus ascertained, was found to equal about half the value obtained by direct multiplication of the length (l) by the width (w) by the height (h). From this and purely theoretical considerations, it became apparent that the volume of the protruding portion of the lesion represented one-

half of a spheroid. Thus a formula, $\frac{\frac{4}{3}\pi\frac{l}{2}\times\frac{w}{2}\times h}{2}$ or $0.5236 \times l \times w \times h$ could be employed. To test the validity of this assumption, 20 lesions were measured and their volumes calculated with the aid of the above formula. Impressions were then made of the same twenty lesions and their actual volumes ascertained by filling these impressions with water. Then mean volumes obtained in both ways were compared with the aid of the paired *t*-test. There was no significant difference between these mean volumes ($0.1 > P > 0.2$). Henceforth the formula $0.5236 \times l \times w \times h$ was employed for calculating volumes of all lesions.

G. Statistical Methods

The statistical methods employed in the analysis of the experimental data involved the paired and non-paired *t*-tests, analysis of variance, Hartley's sequential test, Scheffé's test, and the test for linearity of regression. Hartley's test was the only one employed when the samples were equal or nearly equal in size. If the samples were frankly unequal, Scheffé's test as well as Hartley's test were usually used. The latter method, much more sensitive than the former, is as a sequential test best applicable for comparisons of equal samples; however, it may be applied to unequal samples if the average number in samples (n_0) is properly calculated. Snedecor (1959) suggests the following formula for calculation of this average number: $n_0 = \frac{1}{a-1}\left(n_. - \frac{\Sigma n_i^2}{n_.}\right)$ where a = number of samples; n_i = number in individual sample; $n_. = \Sigma n_i$. In the light of the great variance characteristic of most of our samples, the rather generalized and relatively insensitive Scheffé test failed to demonstrate certain differences the presence of which could be shown with the aid of Hartley's method in which the average number in samples, n_0, was employed. While in some instances the differences demonstrated by this latter method might have been less significant ($P > 0.05$) than the test would indicate, according to experts the nature of the data justified its employment.

The following standard sources were consulted in reference to the various statistical methods: Dixon and Massey (1951), Federer (1955), and Snedecor (1959). The last text was the one employed most extensively.

OBSERVATIONS

Pathogenicity of the Parasite Strains to the Natural Hosts

So far as can be ascertained from reports in the literature, Jones' Barn strain of *T. gallinae* is the most virulent to the natural hosts, pigeons. The parasites of this strain cause serious pathological changes in the esophagus, crop, and liver, with occasional involvement of other viscera. All the experimentally infected birds succumb within about 8 days. According to our findings, which coincide with those of Stabler (for review, see Stabler, 1954), the liver is infiltrated with yellowish, caseous lesions accompanied by a considerable amount of necrosis. The walls of the esophagus and crop are covered with lesions and contain a large amount of purulent matter mixed with mucus. The organs exhibit numerous perforations and in some regions the wall of the esophagus is completely destroyed. On microscopic examination smears of the infected parts of the liver and the esophageal and crop contents reveal numerous active flagellates. Sections of the infected liver show massive invasion of all its parts by the flagellates. The cytoplasm of some liver epithelial cells contains clearly recognizable trichomonads, suggesting the invasion of the cells by the parasites.

Little is known of the pathology caused in pigeons by TG strain. Kupferberg has not described the morbid changes, if any, in the host from which he isolated this strain.

The Lahore (YG) and Amherst (AG) strains did not seem to cause any obvious pathological changes in the pigeons from which they were isolated. Stabler, whose experience with various strains of *T. gallinae* is as extensive as that of any investigator, considers YG strain as one of the mildest. The experimentally infected pigeon from which we isolated this strain did not show any obvious symptoms and no gross pathological changes could be found on autopsy. In the bird naturally infected with the AG strain, the only evidence of infection, beyond the presence of trichomonads, was a somewhat increased amount of mucus in the upper digestive tract. No gross pathology was observed during autopsy.

According to Kupferberg (personal communication): "*T. vaginalis* (strain) No. 1 is the original strain employed by Trussell in inducing experimental trichomoniasis in women. Strain No. 3 was isolated from an active case of the disease." Since we do not have any information concerning the symptoms or pathology found in the patient from whom strain No. 3 (TV 3) was originally isolated, it is impossible to reach any conclusion concerning its relative pathogenicity. We are somewhat more fortunate in reference to strain No. 1 (TV 1), because the report of Trussell and Plass (1940),

subsequently discussed by Trussell (1947), included a detailed description of symptoms and gross pathological changes observed in experimental infections caused by the introduction of this strain (see Kupferberg above) into the vagina of volunteer patients. In the 9 (out of 28 infected) women in whom the parasites were established successfully, there was a whole spectrum of response ranging from transient and scanty vaginal discharge with practically no inflammatory changes in the vaginal mucosa and with no discomfort to the patient, to copious purulent bubbly discharge accompanied by reddening of the vaginal mucosa and considerable discomfort to the woman. In the latter instance the symptoms persisted for many weeks. All indications are that a year or so after its isolation in culture, strain TV 1 was not excessively pathogenic to the natural hosts. This evaluation must of course be viewed with caution, because the strain had been maintained in vitro prior to its employment in the experimental inoculations (for data on attenuation of strains maintained in culture see below).

TVC strain came from a case of active vaginitis. The patient from whom this strain was isolated had a long history of chronic trichomoniasis with periodic recurrences of acute phases. During these phases the woman complained of much discomfort and had copious purulent discharges. No record is available concerning the cytopathological changes, if any, that accompanied this infection.

TVC_1 strain was isolated by Jacobs from a case of exceptionally acute vaginitis.

It has been mentioned by several workers, including Bos (1934) that strains of *T. gallinae*, after having been maintained in culture for some time, may lose their infectivity to the natural susceptible hosts; however, such strains remain capable of producing lesions in experimentally infected mice. As far as *T. vaginalis* is concerned no adequate data are available dealing with the decrease in infectivity to humans of strains maintained in vitro, nor is there any information correlating such attenuation in the natural and experimental hosts. Judging from the previously cited experiments of Trussell and Plass (1940), there is good evidence that, after having been grown for many months in culture, TV 1 strain continued to be infective to 9 out of 28 female patients at least.

Growth Curves of the Parasite Strains

Preliminary experiments suggested that among the strains of *T. gallinae* those which appeared to exhibit the highest growth rate in the test tube were also most virulent to mice. The situation seemed to be quite the reverse among the strains of *T. vaginalis*. Approximate estimates of the multiplication rate of the avian parasites in the lesions of mice indicated that in this instance also the highest rates were characteristic of the most virulent strains. A similar relationship seemed to exist in reference to the human genital species.

To test the preliminary findings growth curves were prepared of the strains which were being employed in the majority of our experiments.

Inspection of the upper graph in figure 1 reveals the fact that among the strains of *T. gallinae* JB grows somewhat faster than TG which has, in turn, a considerably higher division rate than YG. The generation times calculated from the curves were three and one-half hours for JB; three hours fifty minutes for TG; and five hours fifty minutes for YG. These findings confirmed fully the preliminary observations concerning the positive correlation between the rates of growth which the strains exhibit in vitro and the degree of their virulence to mice (see below) as well as to pigeons, the natural hosts.

The growth curves of *T. vaginalis* (figure 1, lower graph) indicate that TV 3 is the fastest and TVC_1 the slowest growing strain. The growth rate of TVC falls between those of the other two. The generation times of the three strains were 4 hours 25 minutes for TV 3; 5 hours 10 minutes for TVC; and 6 hours for TVC_1. Thus the data confirmed our impression that unlike those of the avian trichomonad, the strains of *T. vaginalis* exhibit growth rates in vitro that are inversely proportional to their virulence in the experimental hosts (see below).

Serial Passages of the Parasites in Mice

Reports in the literature as well as our own preliminary experiments indicated that mice were relatively susceptible hosts for trichomonads and that the subcutaneous tissue provided them with an adequate site in which to multiply. It is accepted generally that only through direct serial transfers of parasites from

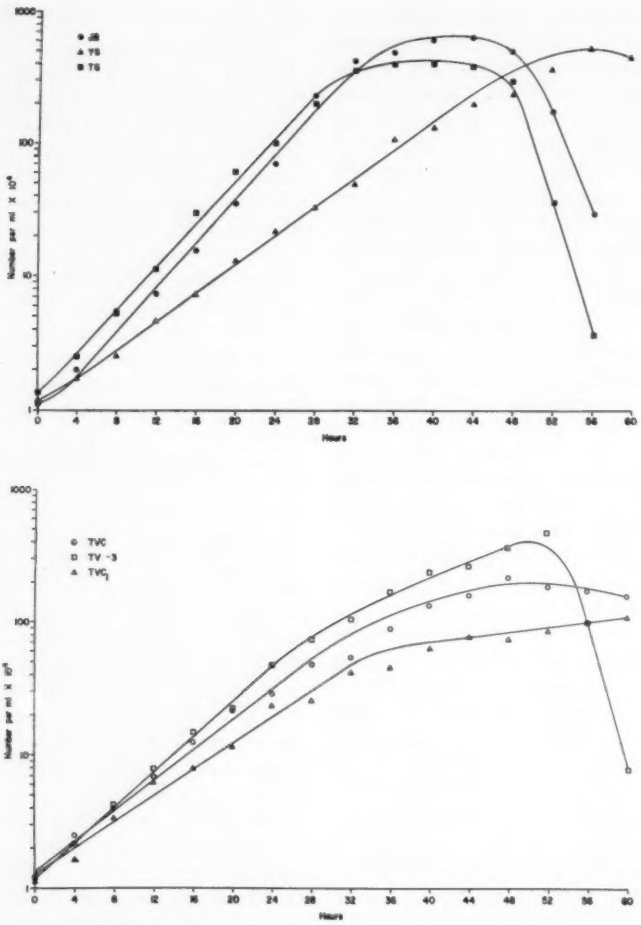

FIGURE 1. Growth curves of three strains of *Trichomonas gallinae* (upper graph) and of three strains of *T. vaginalis* (lower graph) on BBL fluid thioglycollate with 5 percent normal horse serum.

one host to another can one establish unequivocally the susceptibility of the host and the suitability of the site. The considerable degree of susceptibility of mice to the avian species was indicated by Bos (1934) who found that intraperitoneal injections were most successful for mouse-to-mouse transfers. By employing this route of inoculation, the latter author could accomplish six passages; the seventh passage gave negative results. Many factors might have accounted for the failure to maintain the strain indefinitely in mice.

Since serial transfer experiments involving lesions of the type produced by trichomonads in mice are quite time consuming, it was decided to employ one strain of one of the two species in this experimental series. JB strain of *T. gallinae* was chosen because of its apparently high division rate in the lesions. The more numerous are the active flagellates in a single lesion, the fewer abscesses need to be used to insure a sufficient quantity of parasites for a large number of inocula. Starting with the second passage, the procedure employed in the experiments involved the opening of 5-day lesions under sterile conditions, suspending their contents in FTS 5 (with 1000 units of penicillin and 500 micrograms of dihydrostreptomycin per

ml), counting the number of flagellates per ml and adjusting this number with the same medium to about 1.7×10^6 per ml. One-half ml aliquots of the suspension were injected into mice.

TABLE I. *Serial passage of JB strain of* T. gallinae *in mice.*

Passage	n* Intact lesions	n* Open lesions	$\bar{x}$**	s***
I	39	10	110.72	58.76
II	17	6	149.79	75.38
III	11	4	218.44	97.42
IV	11	4	200.89	91.95

* Total number of lesions; intact lesions = number of lesions that could be measured.
** Mean volume in mm^3 of five day lesions.
*** Sample standard deviation.

The data derived from the passage experiment are summarized in table I. It is immediately evident from this table that even with JB strain the total volume of material suitable for the inocula (i.e., containing a sufficiently high number of trichomonads) decreased with each successive passage, thus reducing the number of treatments. Part of the reason for this decrease in the amount of available material lies in the fact that even by the fifth day a relatively high percentage (up to more than 25 percent) of the lesions are open and draining. It was noted that the number of abscesses which open "spontaneously" on the fifth day following inoculation was higher among the lesions produced by injections of suspension of lesion contents than among those caused by trichomonads maintained in vitro. Possibly the purulent contents of the lesions caused additional irritation.

The data indicate that mean volume of 5-day lesions, and thus the virulence of the parasites to mice (see below), appears to increase somewhat on the second and third passages and to level off by the fourth. The analysis of variance gave an F value which indicated the existence of highly significant differences among the four groups. It could be demonstrated with the aid of Scheffé's (F at 5 percent level) and Hartley's* ($n_0 = 17.23$) tests that the mean volumes of lesions obtained in the third and fourth passages of the parasites in mice were significantly different from the mean volume of lesions calculated for the first passage. According to both tests the mean volume of abscesses in the second passage was not significantly different from that in the first. However, while with the aid of Scheffé's test no significant differences could be demonstrated between the volume of lesions obtained in the second and either the third or the fourth passage, such a difference was found between the second and the third passage through the application of the sequential method. Although the values arrived at in Hartley's test failed to show a significant difference between the second and the fourth passage they were very close to indicating its presence.

Since JB strain can be maintained in mice through four serial subcutaneous passages (no further passages have been attempted as yet) and since its pathogenicity to these experimental hosts appears to increase as the result of these passages, it follows that, for this strain of *T. gallinae* at least, mice represent relatively susceptible hosts and subcutaneous tissue a relatively suitable site.

For the present time we are satisfied that since the strains of both the avian and human species exhibit many basic similarities in their effects on mice, these hosts are equally or about equally suitable for all these parasites. However, we hope to test this hypothesis further in the future.

Gross Pathology in Mice (Plates I and II)

1. *T. gallinae* (figs. 1–6). The most common type of lesion caused by subcutaneous inoculation of the parasites into mice is represented by a hard abscess which protrudes beyond the body surface (figs. 3–6). The abscesses are filled with a more or less compact mass of purulent matter which contains numerous motile trichomonads. Except for their size (compare figures 3 and 4 with figures 5 and 6), the lesions produced by the various strains look very much alike. However, the larger lesions, caused by the more virulent strains, have a tendency to break open and to drain after about 1 week; often by the end of the second week only a small number of intact abscesses remain. On some occasions the primary lesion may close and a secondary abscess develop; on other occasions the open lesion may heal completely within several days or weeks, and the scar tissue alone marks the site of the original abscess; sometimes the drain-

* In this and in all other instances which involved the comparison of unequal samples n_0 was calculated according to the formula given by Snedecor (see Materials and Methods).

ing lesions fail to heal for extended periods. The opening of the lesions is undoubtedly aided by the mouse which scratches the irritated area of the skin with its hind paw, or rubs its side against the walls of the cage; however, the very stretched skin covering a large abscess may rupture quite easily without much mechanical aid.

Among lesions produced by the most virulent strain, JB, particularly within a relatively short time after its isolation (2 to 6 months), another type may be found on occasions. This second type is represented always by a very large swelling and feels rather soft to the touch (fig. 2). The lesion is filled with gas and fluid. Small islands of yellowish tissue are seen on the walls of the otherwise nearly empty space. The fluid is teeming with active parasites. On rare occasions this gas-filled type of lesion may spread throughout the subcutaneous tissue of the host (fig. 1). Mice thus affected exhibit generalized edema and their body is soft and spongy. This type of lesion resembles the one which Newton, Reardon and DeLeva (1960) reported in connection with the subcutaneous inoculations of TVC_1 strain of *T. vaginalis* into

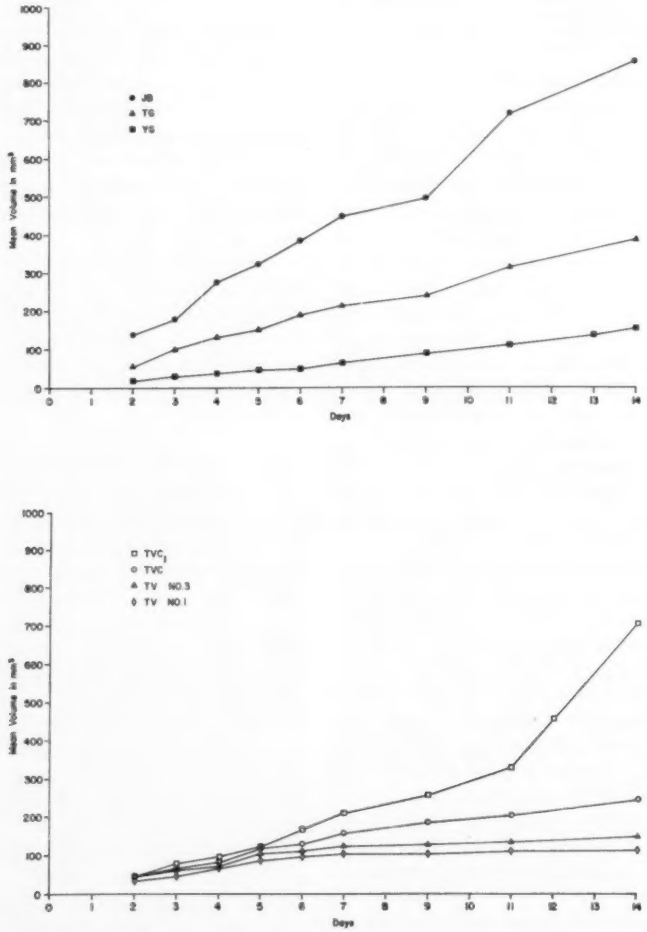

FIGURE 2. Mean volumes of lesions produced in C57 B1/6 mice by subcutaneous inoculations of three strains of *Trichomonas gallinae* (upper graph) and of four strains of *T. vaginalis* (lower graph).

germ-free guinea pigs.

2. *T. vaginalis* (figs. 7–10). In their general appearance the lesions produced by the strains of the human genital trichomonad do not differ materially from those caused by *T. gallinae*. The differences among the abscesses produced by various strains are also quantitative rather than qualitative (compare figures 7 and 8 with 9 and 10). In all instances the abscesses are of the hard type filled with trichomonad-containing purulent material. The spreading gas-filled lesions observed with JB strain of *T. gallinae* were not found in connection with any of the strains of *T. vaginalis*, although some of the abscesses caused by TVC_1 approached them in appearance.

Just as in the case of the lesions produced by *T. gallinae*, the larger abscesses caused by the more virulent strains of *T. vaginalis* often open and drain during the 2nd week following inoculation. Some of the lesions produced by the very pathogenic TVC_1 strain usually started to rupture before the end of the first week, and only very few intact abscesses were left for measurement at the end of the second.

In most respects our observations concerning the gross appearance of the subcutaneous lesions caused by inoculation of trichomonads into mice agree with those reported by Schnitzer et al (1950) and Iwai (1957). Since the former authors did not deal with a strain of *T. gallinae* the virulence of which equalled or approached that of the JB strain, it is not surprising that they failed to find any of the gas-filled, spreading type of lesions.

Comparison of Pathogenicity of the Parasite Strains to Mice

Since all the strains of both species have been cultivated under nearly identical conditions in the absence of any foreign organisms and since they have been inoculated into the same site in a pure-bred strain of mice, the differences in virulence which these strains exhibit in such mice probably reflect true genetic differences among the parasites.

Figure 2 represents graphs relating to time the mean volumes of subcutaneous lesions produced by three strains of *T. gallinae*, JB and TG in January 1957 and YG in May 1957* (upper graph) and by four strains of *T. vaginalis*, TVC, TV 3, and TV 1 in January and May, 1957 and TVC_1 in March and April 1959 (lower graph). (See table III for explanation of more than one date for all strains of *T. vaginalis*). The relationship between the mean volumes and time was checked for linearity of regression (see tables II and III for data). The values for F variance ratio obtained from the standard test were significant for all strains. It could be thus concluded that the relationships were curvilinear. In view of the multiplicity of factors, involving both host and parasite physiology, which undoubtedly affect host response, this situation is not at all surprising. Since curvilinear relationships are very difficult to analyze statistically, no attempt was made either at fitting the curves or at analysis of the data in terms of regression and correlation.

It is evident that among strains of *T. gallinae* represented in the graph, JB produces the largest lesions. The slope of the curve also suggests that the rate of increase of the lesions caused by this strain is the highest. The mean volumes of abscesses resulting from inoculations of TG strain are smaller than those characteristic of infections with JB. YG causes by far the smallest lesions. Table II represents the data which were used in the statistical analysis of the results.

Analyses of variance of the data obtained on the 2nd to the 14th days following inoculation gave F values which indicated the existence of highly significant differences among the mean volumes of lesions at all these times. Hartley's sequential tests, for comparison of the volumes obtained between the 2d and 11th days inclusive, proved that on each of these days the volumes could be ranked according to their size as follows: JB > TG > YG. Despite the pooling of data from three experiments, the total number of measurable lesions produced by JB on the 14th day following inoculation was much smaller than of those caused by the remaining strains. Still, both Scheffé's (F at 5 percent level) and Hartley's ($n_0 = 17.53$) tests indicated that on this day also JB > TG > YG.

A comparison with the aid of the non-paired t-test, of the mean volumes of lesions produced by AG (Exp. 1/24/59) and YG (Exp. 12/12/58) strains on the 5th and the 14th days following inoculation (data not included in

* Since no significant difference was found between the mean volumes of lesions produced by YG strain in January and May, 1957 (see Table IV below), the data obtained in the May experiment, which involved a larger number of inoculations were employed in the statistical comparison of the three strains.

figure 2 or table II) indicated the absence of any significant differences between them (5th day – $0.1 > P > 0.05$; 14th day – $0.5 > P > 0.4$).

It is evident from the foregoing data that the mean volumes of lesions produced by the strains of *T. gallinae* in mice reflect the degree of pathogenicity of these strains to their natural avian hosts (see above).

TABLE II. *Mean volumes of subcutaneous lesions produced in mice by three strains of* T. gallinae.

Day after inoc.	Strain	n	$\bar{x}$*	s
2	JB	25	139.72	95.69
	TG	25	54.37	40.84
	YG	26	20.45	13.51
3	JB	25	177.54	118.45
	TG	25	99.22	80.31
	YG	26	28.48	11.80
4	JB	25	274.29	185.30
	TG	25	132.37	111.05
	YG	25	45.50	24.45
5	JB	25	323.70	244.26
	TG	25	152.64	94.71
	YG	25	48.19	26.75
6	JB	21	383.10	315.67
	TG	25	190.04	100.00
	YG	25	63.43	31.96
7	JB	21**	448.77	314.74
	TG	21	214.44	145.29
	YG	24	86.15	44.99
9	JB	21**	492.04	262.20
	TG	21	238.62	113.15
	YG	24	109.69	59.28
11	JB	20**	716.00	424.20
	TG	21**	314.64	253.60
	YG	24	134.48	77.20
14	JB	11**	850.82	706.56
	TG	20**	387.63	280.15
	YG	24	150.09	86.85

* In mm^3 in this and the following tables.
** Since the lesions caused by JB and TG strains, particularly by the former, show a tendency toward opening during the second week following inoculation the numbers, *n*, of lesions represent the results of two or three experiments. Pooling of treatments was preceded by testing the homogeneity of data obtained in the separate experiments, on the particular days for which the measurements were to be pooled, by means of the non-paired *t*-test in the case of two and by *F*-variance in the case of three sets of measurements. In all instances the *t* values and *F* variance ratios were not significant at the five per cent levels (in most instances $P > 0.3$ or 0. 5).

The lower graph in figure 2 pictures the relationships among the mean volumes of lesions produced in mice by TV 1, TV 3, TVC, and TVC_1 strains of the human genital trichomonad. The differences among the strains of this species appear to be less dramatic than among those of *T. gallinae*. However, it is evident that in reference to the size and the growth rate of lesions TVC_1 exceeds by far the remaining three strains. TVC is different in the above respects not only from TVC_1 but also from TV 1 and TV 3 which appear to be similar to each other. As may be seen from the graph, the differences in the mean volumes of lesions caused by the several strains of *T. vaginalis* do not usually become clear until about the sixth day following inoculation; from that time they may be easily observed if present at all.

The curves pertaining to strains TV 1, TV 3 and TVC seem to resemble one another in all their segments. On the other hand, the last part (starting with 11 hours) of the curve representing lesions produced by TVC_1 differs from the remaining three. In both series of experiments involving this strain, the lesions started to open quite early (by the sixth or seventh day following inoculation up to 14 percent were open and draining). By the 11th day only about 35 percent of abscesses were still closed; however, over 80 percent of the lesions which did not open by that time remained intact for the rest of the experiment. These latter lesions continued to increase in volume at a rate much higher than that observed up to the 11th day. It is possible that after a certain period of time the host defenses are weakened and thus the parasites may spread at a faster rate, similar to the one observed in JB strain of *T. gallinae*.

Table III represents the data employed in the statistical analysis of the results obtained after inoculation of mice with the several strains of *T. vaginalis*.

During the earlier stages of this investgation we were dealing with only three strains of *T. vaginalis*, TV 1, TV 3, and TVC. The figures representing the mean volumes of lesions up to about the 6th or 7th day following inoculation did not appear on visual inspection to be significantly different, particularly in the light of the high variance (see values for *s*). However, it was thought worthwhile to subject the data for all days (2 to 14) to the analysis of variance. The *F* variance ratios were significant for all days (5 percent level, day 2; 1 percent level, days 3 to 14) except the 4th. Consequently the data for each day (except the 4th) were subjected to Hartley's test.

The following results were obtained for the 2-, 3-, and 5-day lesions: TVC, TV 3, TV 1. Thus, it became evident that even during the early stages of infection TVC strain produced larger lesions than TV 1, but that TV 3, a strain of apparently intermediate virulence, caused abscesses which were not significantly different from those resulting from inoculations of the remaining two. Starting with the 6th

TABLE III. *Mean volumes of subcutaneous lesions produced in mice by four strains of* T. vaginalis.

Day after inoculation	Strain	n*	$\bar{w}$	s
2	TVC_1	44	46.35	22.25
	TVC	44	45.88	29.19
	TV 3	44	47.11	37.14
	TV 1	47	32.63	20.68
3	TVC_1	43	78.08	47.86
	TVC	44	66.03	41.05
	TV 3	44	63.23	38.36
	TV 1	47	44.99	23.30
4	TVC_1	42	96.63	49.68
	TVC	44	80.56	53.82
	TV 3	44	71.72	45.74
	TV 1	47	67.31	38.22
5	TVC_1**	56	123.66	68.12
	TVC	44	119.20	61.88
	TV 3	44	104.84	51.43
	TV 1	47	85.75	38.84
6	TVC_1	51	167.85	82.24
	TVC	44	131.25	49.01
	TV 3	44	111.74	52.33
	TV 1	47	96.47	36.85
7	TVC_1	38	212.13	141.29
	TVC	44	158.58	65.83
	TV 3	44	124.77	60.18
	TV 1	47	104.03	43.79
9	TVC_1	28	259.68	165.36
	TVC	44	187.11	72.86
	TV 3	44	130.01	54.33
	TV 1	46	104.70	38.32
11	TVC_1	20	331.07	209.92
	TVC	43	204.74	76.57
	TV 3	43	136.46	65.92
	TV 1	45	112.76	41.53
14	TVC_1	13	709.77	485.07
	TVC	43	248.67	137.41
	TV 3	41	151.88	74.34
	TV 1	44	113.59	57.45

* Except when indicated otherwise, the total number of lesions (n) represents two replicate experiments, which, when compared by the non-paired t-test, failed to show any significant differences.

** The total numbers of lesions (n) involving strain TVC_1 between the fifth and fourteenth days are all based on three replicate experiments, which when tested for heterogeneity gave non-significant variance ratios, F.

day the mean volumes of lesions produced by the three strains could be ranked as follows: TVC, TV 3, TV 1. The presumably most virulent strain, TVC, became significantly different in its pathogenicity to mice than either TV 3 or TV 1; however, the latter two strains could not be distinguished by the sequential test.

The mean volumes of lesions produced by TV 3 and TV 1 between the 6th and the 14th day following inoculation were next compared by non-paired t-tests. These tests which took into account less variance, indicated the presence of significant differences between the two strains on the 9th to 14th days (the least significance $P < 0.05$). The 6th ($0.2 > P > 0.1$) and 7th ($0.1 > P > 0.05$) day measurements showed no such differences.

Since, as will be seen from the experiments which follow, there is a certain amount of attenuation of the strains of the genital trichomonad of man on cultivation in the test tube, it is quite difficult to say whether TV 1, originally isolated in 1939, and TV 3 strain, originally isolated in 1945, were or were not equally pathogenic to their natural hosts at the time of their isolation. It is even possible that they might have been not less, perhaps even more, pathogenic than TVC strain, originally isolated in 1956. However, our experiments suggest that the attenuation of pathogenicity among trichomonad strains having reached a certain level (generally proportional to their original virulence), continues at that level for a long period of time. Since in the light of past experience it may be assumed that both TV 1 and TV 3 reached this level some time ago, it is quite possible that the relative degrees of virulence of these strains to mice reflect their relative pathogenicity to the patients from whom they were originally isolated.

In the early spring of 1959 we received C_1 (TVC_1) from Dr. Leon Jacobs with his permission to test the pathogenicity level of this strain according to our method.

The mean volumes of 6-day subcutaneous lesions produced by all four strains of *T. vaginalis* dealt with in this report were subjected to analysis of variance. When, as might have been expected, the F variance ratio was significant, the data were further analyzed with the aid of Hartley's test ($n_0 = 46.44$). According to the results obtained from this test TVC_1 produced larger lesions, was presumably more virulent to mice, than the remaining three strains. The latter could be ranked as follows in reference to the size of abscesses which they caused: TVC, TV 3, TV 1. Despite the sizable values for variance, the sequential test confirmed statistically our observations concerning the relative virulence of the several strains, in reference to 6-day lesions at least. Furthermore a comparison, with the aid of the t-test, of the 7 to 14-day lesions produced by TVC_1 and TVC indicated that on these days also significant differences existed between the two strains (the least significance $P < 0.05$).

It will be noted that the data relating to TVC strain employed in the above test were obtained in experiments performed in the winter and spring of 1957, while those pertaining to TVC_1 were derived from inoculations made in the spring of 1959. Although the former strain was isolated about 6 months prior to the latter,

it had been maintained in culture for a much shorter period before being injected into mice. Consequently if attenuation of the strains grown in vitro were to be considered, TVC should have been attenuated less than TVC_1; even under those circumstances the former could be proved significantly less pathogenic to mice. As much as there might have been some doubt in reference to the results obtained with strains TV 1 and TV 3 which had been maintained in culture for a relatively long time, the validity of the data concerning TVC_1 and TVC cannot be questioned.

In many reports dealing with pathogenicity of *T. vaginalis* and *T. gallinae* and even in some of those that are based on inoculation into mice, statements may be found which clearly indicate or at least imply that the avian parasite exceeds the human genital trichomonad in pathogenicity. It was felt that such statements could be considered valid only when applied to the particular strains employed by the various investigators, for even a cursory inspection of our data indicated that some of the strains of *T. gallinae* used in the present experiments were less virulent to mice than most or all strains of *T. vaginalis* with which they were compared. An objective evaluation of the results, however, could be achieved only through statistical analysis. Consequently the data were subjected to appropriate tests. All the measurements employed in these tests are presented in tables II and III.

Measurements of the 6-day lesions involving three strains of *T. gallinae* and four of *T. vaginalis* were tested for significant differences among the seven groups by means of the analysis of variance. The variance ratio, F, was significant; thus it could be concluded that such differences were present. Since the sizes of samples (n) involving the strains of the avian species were smaller than those involving the strains of the human genital trichomonad, Scheffé's test (F at 5 percent level) was applied to the data first. However, this generalized and relatively insensitive test failed to demonstrate many of the intra-specific differences among the strains which were indicated previously through the application of the sequential method to the data. Hartley's test ($n_0 = 36.11$) was then applied to the data. When the variance of JB strain was included in the s^2 value, the results were as follows:

JB, TG, TVC_1, TVC, TV 3, TV 1, YG. While capable of demonstrating a few differences that were not shown with the aid of Scheffé's test, the sequential method did not prove to be much more powerful. However, when variance of JB was excluded from the calculations, the results, TG, TVC_1, TVC, TV 3, TV 1, YG, approached more closely those obtained through the analysis of the strains of each species.

The shape of the curves represented in figure 2 suggested the need for ascertaining whether JB and TVC_1 strains were significantly different throughout the entire duration of the experiments. When the daily measurements of the lesions produced by these two strains were compared with the aid of the t-test, it became apparent that the abscesses caused by JB strain were very significantly larger than those resulting from the inoculation of TVC_1 up to the 11th day following the treatment (2d to 9th day $P < 0.001$, 11th day $0.001, < P < 0.005$). However, the test indicated that the 14-day lesions produced by these strains were about equal in volume ($P > 0.5$). The possible significance of the sudden increase in volume of the TVC_1 lesions has been discussed elsewhere in this report.

The t-tests also indicated that TV 1 produced significantly larger lesions than YG on all days following inoculations (from $P < 0.001$ to $P < 0.05$, depending upon the day on which the readings were taken).

The foregoing data suggest that there are strains of *T. vaginalis*, TVC_1 for example, which after being kept in culture for approximately the same length of time as the most virulent strain of *T. gallinae*, JB, may at some time following inoculation, cause lesions as large as those produced in mice by the latter strain. It seems also clear that the least virulent tested strain, TV 1, of the human genital trichomonad, which has been kept in culture seventeen years longer than some of the strains of the avian species, for instance YG, has remained more virulent to mice.

The reports of Stabler (1948, 1950, 1951, 1954), Stabler and Matteson (1950), and Stabler and Herman (1951) indicate that the majority of the strains of *T. gallinae*, restricted to the upper digestive tract, cause relatively little damage to the natural hosts. From the observations published by various workers as well as on the basis of the present investigation

one can conclude that the most virulent strains of *T. gallinae* cause more severe pathological changes in the natural and experimental hosts than most if not all strains of the human genital trichomonad. On the other hand, all the available data suggest that there are many strains of the avian trichomonad which are actually less pathogenic than a number of strains of *T. vaginalis*.

Attenuation of Strains Maintained in Vitro

It became apparent in the course of this study that the volume of lesions produced in mice by strains of *T. gallinae* and to a less extent of those caused by strains of *T. vaginalis* decreased in direct proportion to the length of time the parasites were maintained in culture. The progressive attenuation of virulence in two strains of the avian species and in one of the human genital trichomonad is represented in table IV.

TABLE IV. *Attenuation of* T. gallinae *and* T. vaginalis *maintained in vitro.*

Species	Strain	Date of inoculation	Day after inoculation	Lesions		
				n	$\bar{x}$ (volume)	s
T. gallinae	TG	Jan. '57	6	25	190.04	100.00
			14	21	387.63	304.99
		May '59	6	26	80.52	25.93
			14	20	136.65	56.79
		Jan. '57	6	18	51.15	25.06
			14	18	157.62	105.61
		May '57	6	25	48.19	26.75
			14	25	150.09	86.85
	YG	May '58	6	16	27.81	24.48
			14	16	36.19	36.17
		Jan. '59	6	16	9.63	7.95
			14	16	20.27	17.70
		June '59	6	23	9.28	8.12
			14	23	10.97	10.01
		Dec. '59	6	20	13.16	10.35
			14	20	18.04	17.02
T. vaginalis	TVC	Jan. '57	6	15	138.29	67.71
			14	15	274.88	194.12
		May '57	6	30	127.73	37.32
			14	28	234.62	96.17
		Apr. '59	6	33	96.01	59.15
			14	20	193.02	116.12

The data obtained from measurements of 6- and 14-day lesions produced by YG strain of *T. gallinae* were subjected to analyses of variance. Since the F ratios were significant in both instances, the data were further analyzed with the aid of Hartley's test ($n_0 = 19.84$). It could be demonstrated that the mean volumes of lesions obtained in the earliest experiments (January and May 1957), which showed no difference ($P > 0.5$), were significantly larger than those measured in all four subsequent inoculations on both the 6th and 14th day following the treatment. No significant differences were found among the data derived from the remaining experiments; however, as might have been expected, significant differences could be found in many instances when the mean volumes of 6- or 14-day lesions obtained in these experiments (see table IV) were compared with the aid of the non-paired t-tests.

The data pertaining to experiments with TG strain of *T. gallinae* were analyzed by non-paired t-tests. According to these tests the lesions obtained in the first experiment (January 1957) were significantly larger than those obtained in the second (May 1959) on both the 6th ($P < 0.001$) and the 14th ($0.001 < P < 0.005$) day following inoculation.

As far as strain TVC of *T. vaginalis* was concerned, it could be ascertained with the aid of t-tests that no significant differences existed between the mean volumes of 2- to 14-day lesions resulting from the first (January 1957) and the second (May 1957) series of inoculations. The pooled data were then compared with the measurements obtained in the latest experiment (April 1959). Significant differences ($P < 0.001$ to $P < 0.05$) were found between the two sets of measurements made at the usual time intervals between the 2d and the 14th day following inoculation.

The foregoing statistical analyses suggest that in the case of *T. vaginalis* attenuation of virulence is less dramatic and requires more time than in that of *T. gallinae*. It seems apparent also from this and other (see above) evidence, that having reached a certain level of pathogenicity to mice the trichomonad parasites remain at this level for a considerable period of time. It could be argued that strains of the avian trichomonad, which in some of our experiments appeared to be less virulent to mice than those of the human genital parasite, could have been actually more pathogenic if tested very much sooner after their isolation. However, our conclusions appear to be supported by the following facts: (1) It is evident from table IV and from the statistical analysis of the data that there was no significant change in virulence to mice

in either TVC strain of *T. vaginalis* or YG strain of *T. gallinae* between January and May 1957. (2) It was observed that the degree of attenuation of pathogenicity to mice of AG strain, which is nearly as mild as YG, was relatively small between the second week and the fifth month following its isolation. (3) As indicated in the previous section, TV 1 strain of the human genital trichomonad was maintained in culture 17 years longer than YG or AG strains of the avian parasite and in view of our experience should have been attenuated to a greater degree than either of the two latter strains. Still, it was proved to be significantly more virulent to mice.

In the light of all the data it appears that a considerable amount of information concerning the relative pathogenicity of strains of the avian and the human genital trichomonads can be derived from inoculations of strains maintained in vitro for different periods of time; however, it is clear that even on the intraspecific level more precise evaluation can be achieved through inoculations into mice of strains which have been kept in culture for equivalent or nearly equivalent times. As far as the interspecific comparisons are concerned it would seem best to base them on experiments with strains that have been maintained in culture for relatively short periods of time. Such experiments are now in progress.

Influence of Agar and Other Substances on Development of Lesions

It was noted early in the course of this investigation that when TG strain of *T. gallinae*, maintained on STS without agar, was injected into mice relatively few inoculations resulted in abscesses. Those that were produced seemed to be very much smaller than the ones seen after the introduction of the same strain of parasites grown in an agar-containing medium. When the trichomonads were returned to STS with agar and inoculated into mice in this medium the resulting abscesses appeared to form with the usual frequency and were of about normal size. Similar results were obtained when an appropriate amount of agar was added just prior to inoculation to the suspension of organisms maintained for a few transfers in the absence of this substance.

Thus, it would seem that agar which, as the control experiments indicated, could by itself cause no apparent lesions, acted as an irritant affecting the parasites, or the host tissues, or both, in such a way as to stimulate the development of abscesses. Anoxia, favorable for the parasites, would at the same time affect detrimentally the host tissues and might be postulated as a condition that could be caused by the presence of agar. Since the host cathepsins would not be able to attack this substance, such a hypothesis appears justified, on theoretical grounds at least. However, it became apparent soon that while agar might very well act as a direct irritant, it seems to play also some indirect role by affecting the physiology of trichomonads grown in vitro in a way which enhances their virulence to mice. This latter assumption was based on the findings summarized graphically in figure 3.

All the individual samples used in the experiments represented in figure 3 involved 30 inoculations of organisms grown on CPLM without agar. The data suggest that the longer a strain of either *T. gallinae* or *T. vaginalis* is maintained on a medium without agar, the fewer lesions it is capable of producing (lower graph) and the smaller are the volumes of such lesions (upper graph). It must be noted that the amount of agar present in the medium is negligible after as few as three serial transfers (1 week); after nine transfers (3 weeks) the concentration of agar is nil for all practical purposes. In view of the foregoing, the presence of this substance in the medium cannot be considered as a factor influencing either the size or the percentage of lesions. Since in many instances only a small number of inoculations produced any lesions, particularly after all but the most virulent strains had been maintained for 3 or more weeks on media without agar, a statistical analysis of the data pertaining to mean volumes was deemed impractical. In the light of the small number of positive readings in each sample the occasional reversals in both the mean volumes and percentages of lesions do not seem too surprising. Undoubtedly more meaningful and statistically analyzable data could have been obtained if each sample were to contain a larger number of injections, but it seemed questionable whether the additional information thus obtained would justify the expenditure of time and money required to handle very large samples.

The data obtained in this series of experi-

ments indicated that the least virulent strain of *T. gallinae*, YG, failed to produce any lesions in thirty inoculations after it had been maintained for 9 weeks on a medium without agar. It could be ascertained also that the relative degree of pathogenicity of the strains of both species was faithfully reflected in the mean volumes and percentages of lesions which they caused on successive inoculations. Probably after longer periods of maintenance on media without agar all the strains could be brought to a level of virulence at which they would produce no lesions in 30 inoculations. However, it is likely that if an infinite number of inoculations were made, an infinite period of time would be required for even the mildest strain to lose completely its capacity for producing abscesses.

In addition to the foregoing another test was performed to provide some further clues as to the nature of the involvement of agar in

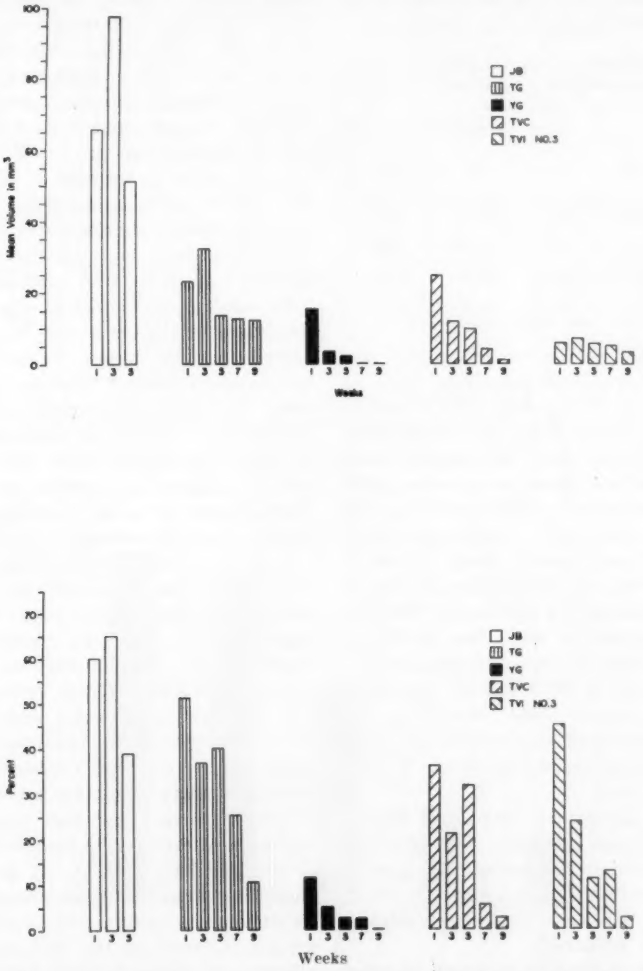

FIGURE 3. Decrease in virulence among three strains of *Trichomonas gallinae* and two strains of *T. vaginalis* as reflected by the decrease of the mean volumes (upper graph) and the percentage (lower graph) of 12-day subcutaneous lesions produced by inoculations into C57 B1/6 mice of these strains maintained for various periods on CPLM without agar. Since JB strain isolated in August 1956 was lost (see Materials and Methods) during the course of experiments represented in figure 3, only three series of inoculations were made.

the production of lesions. TGNA strain, maintained on STS without agar since March 1955, was carried through three transfers on STS with agar. TG strain (parental strain of TGNA), maintained since its isolation (1947) on media with agar (in our laboratory since 1955 on FTS 1), was also carried through three transfers on STS with agar. The two strains were subsequently injected into mice in the latter medium. Table V represents the data obtained from measurements of lesions on the 6th day and the 14th day following inoculation.

TABLE V. *Mean volumes of subcutaneous lesions produced by inoculation of TGNA and TG strains in STS with agar.*

Day after inoculation	Strain	n	$\bar{x}$	s
6th	TGNA	30	67.60	49.96
	TG	26	80.52	25.93
14th	TGNA	23	89.48	31.31
	TG	19	136.65	56.79

An inspection of the results suggests that TGNA strain maintained for several years on STS without agar when returned for a week to a homologous medium with agar caused somewhat smaller lesions than its parental strain which has never been grown on a medium without agar. A statistical analysis employing the *t*-test indicated that while no significant difference could be found between mean volumes of 6-day lesions ($0.4 > P > 0.2$), those of the 14-day lesions showed such a difference ($0.005 > P > 0.001$). It should be noted that no lesions were observed after 14 days following 34 inoculations of TGNA in STS without agar. Furthermore, the initial edema caused by the control inoculations of sterile STS with agar disappeared completely within 48 to 72 hours following treatment.

The latter experiments appear to confirm the conclusion based on results obtained in the series of inoculations summarized in figure 3, for they suggest that agar, in addition to acting as an irritant, has some virulence-stimulating effect upon the parasites.

It seemed of interest to ascertain if other viscous substances such as methyl cellulose, mucin, or gelatin, when added to the medium on which trichomonads were grown and inoculated into mice, would enhance the formation of abscesses. To investigate this problem agar in STS was replaced by each of the three substances (see Materials and Methods for details).

Methyl cellulose, mucin, and gelatin were selected for several reasons. All three substances, particularly the latter two, have been employed in a number of culture media for protozoa and bacteria. Methyl cellulose cannot be attacked by either the enzymes secreted by the parasites or by those found in the host tissues. Thus beyond increasing the viscosity of the medium, and probably slowing down the diffusion of oxygen in the test tube, the substance would appear to be relatively inert both in vitro and in vivo, following its inoculation into mice. Furthermore, methyl cellulose requires energy for and is characterized by $+\triangle V$ during sol-gel transformation. In the light of all its attributes this type of substance could be judged to represent an effective irritant, particularly in tissues of homoiothermic hosts. (It may be noted that injections of sterile STS with methyl cellulose often result in the formation of small swellings which may persist for several days before being absorbed by the mouse tissues; in some instances small residual swellings were observed for 2 weeks). Certain mucins (mucoproteins) are present in the organs normally parasitized by trichomonad flagellates. The growth-promoting influence of gastric mucin has been demonstrated in axenic cultures of intestinal trichomonads (Honigberg, 1958a, b) and endozoic amoebae (Stoll, 1957). This substance has been also shown to enhance the virulence of endozoic amoebae in experimental hosts. Finally mucin would not be readily attacked by host-tissue enzymes. Thus, in addition to causing a certain amount of irritation to the host tissues, this compound could exert some effects upon the physiology of the parasites which might result in their enhanced virulence. Gelatin, undoubtedly susceptible to the action of the proteolytic enzymes in the host tissues, does not appear to be attacked by trichomonads in vitro. It seems to have little if any direct influence upon the growth rate of the parasites; however, its presence in culture could easily slow down oxygen diffusion in the medium. This latter effect would appear to be of little value in the host tissues, even if the substance were not subject to the action of cathepsins. From all viewpoints, gelatin, which also loses energy and is characterized by $-\triangle V$ in the sol-gel transfor-

mation, seems to be of little value either as a direct irritant or as a metabolic agent capable of enhancing virulence.

JB strain of *T. gallinae* was employed in all experiments involving STS with methyl cellulose, mucin, and gelatin. The strain was maintained on the several experimental media for eight serial transfers prior to being inoculated into mice.

The upper half of figure 4 represents graphically the mean volumes of lesions observed at 48 hour intervals between the 2d and the 12th days following inoculation of mice with trichomonads maintained for three serial transfers on STS with agar, methyl cellulose, mucin, or gelatin, as well as on STS devoid of all these substances.* The lower half of the same figure depicts the percentages of 6-day lesions pro-

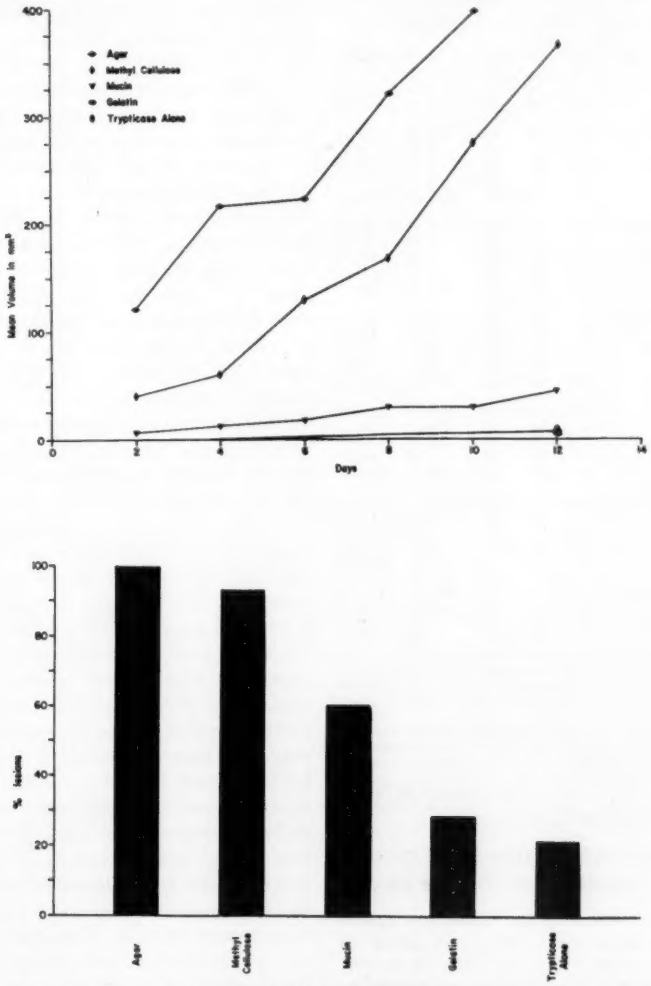

FIGURE 4. Mean volumes of lesions produced in C57 B1/6 mice by subcutaneous inoculations of Jones' Barn (JB) strain of *Trichomonas gallinae* maintained on STS medium with various viscous substances and without any such substance (trypticase alone) (upper graph); and percentage of 6-day lesions caused by this strain grown on STS with such substances (lower graph).

duced by trichomonads maintained on and injected in STS with each of the four aforementioned viscous substances or on STS without any such substance. The data used in the preparation of both graphs and employed in the statistical analysis of the results are summarized in table VI.

TABLE VI. *Mean volumes of subcutaneous lesions produced by JB strain of* T. gallinae *grown with various viscous substances.*

Day after inoculation	Substance	n*			$\bar{x}$	s
		Pos.	Neg.	Tot.		
2	Agar	16	0	16	120.04	66.13
	Methyl cellulose	12	7	19	40.19	23.53
	Mucin	11	17	28	6.27	9.35
	Gelatin	2	26	28	0.15	0.95
	none	0	28	28	0	0
4	Agar	15	0	15	215.97	148.04
	Methyl cellulose	16	3	19	60.21	42.75
	Mucin	11	17	28	11.53	15.46
	Gelatin	5	23	28	3.94	10.02
	none	4	24	28	3.51	9.15
6	Agar	14	0	14	221.87	180.37
	Methyl cellulose	15	1	16	127.96	94.12
	Mucin	17	11	28	17.18	12.03
	Gelatin	8	20	28	4.01	7.90
	none	6	22	28	3.82	8.01
8	Agar**	20	0	20	320.63	214.41
	Methyl cellulose	13	1	14	166.80	116.76
	Mucin	17	11	28	29.03	20.11
	Gelatin	9	19	28	5.49	10.71
	none	7	21	28	5.03	10.21
10	Agar**	14	0	14	398.21	184.60
	Methyl cellulose	13	1	14	274.87	194.16
	Mucin	19	9	28	29.30	19.34
	Gelatin	9	19	28	7.36	15.72
	none	8	20	28	6.97	13.81
12	Agar**	11	0	11	903.38	801.27
	Methyl cellulose	7	0	7	366.72	271.12
	Mucin	20	8	28	45.59	24.15
	Gelatin	10	18	28	9.83	8.53
	none	9	19	28	8.92	14.15

* Number in sample used in calculations included all inoculations, regardless of whether or not they resulted in lesions. In the latter instance the individual volumes were counted as Os.

** In inoculations on STS agar, the number in samples (n) on the 8th, 10th and 12th days represents a pool of two replicate experiments, the results of which showed no statistically significant differences.

NOTE: Decrease in total n in experiments involving STS with agar and methyl cellulose was caused by opening of abscesses.

An inspection of the graphs and of the more complete data represented in the table suggests immediately that gelatin has practically no effect on the formation of lesions. It is evident that both the mean volumes and the percentages of abscesses produced by trichomonads grown on STS with gelatin resemble very closely those resulting from inoculations of parasites maintained on STS which contains no viscous substances. On the other hand it is equally apparent that the lesions produced by trichomonads grown in both of the above media are much smaller than the abscesses caused by the injection of parasites maintained on STS with agar, methyl cellulose, or mucin. The clarity of the results which serve as the basis for the above conclusions seems to eliminate the need for a statistical comparison of the data.

However, it was deemed necessary to test the results obtained in the series of injections of trichomonads grown on STS with agar, methyl cellulose, and mucin. Analyses of variance among the three groups of samples measured on the 2d, 4th, 6th, 8th, 10th, and 12th days following inoculation all gave significant F values. In the light of our previous disappointing experience with the relatively unsensitive Scheffé test in demonstrating differences among samples that exhibit large variances, we were advised to employ the sequential test of Hartley for the comparison of the 6- ($n_0 = 18.48$) and 12- ($n_0 = 12.63$) day lesions. According to this test both 6- and 12-day lesions caused by trichomonads grown on STS with agar are significantly larger than similar abscesses produced by these parasites when methyl cellulose is present in the medium. Furthermore, the volumes of the latter lesions exceed those caused by trichomonads grown on STS with mucin. The foregoing results seem to provide further support for the hypothesis that the role played by agar in stimulating the formation of lesions exceeds that of a simple direct irritant. If it were not so, methyl cellulose, the physicochemical properties of which seem to endow it with all the attributes of an excellent irritant, when present in the inoculum would stimulate the development of lesions at least as large as those caused by agar. The experiments confirm also the assumption as to the lack of any stimulatory effects of gelatin upon the production of abscesses. On the other hand, the influence exerted by mucin appears to be far less than could have been anticipated on theoretical grounds.

* The mean volume of the twelve day lesions produced by inoculations of JB strain grown on STS with agar was so large (see Table 6) in comparison with the remaining volumes represented in the chart, that its inclusion in the graph at the common scale presented technical difficulties. Consequently the graphic representation of the mean volumes of lesions produced by JB strain on STS with agar is limited to 10 days.

It seems clear that the influence which the several substances might have upon trichomonads grown in culture would not necessarily reflect the effects exerted by these compounds on the parasites in vivo. Nonetheless it was felt that some useful information could be derived from a study of the growth response of the flagellates in STS into which were incorporated agar, methyl cellulose, mucin, or gelatin in quantities employed previously in the animal inoculation experiments. Figure 5 represents growth curves of JB strain of *T. gallinae* maintained on STS with each of the four viscous substances as well as on STS ("No agar") to which none of these compounds was added. The generation times (G) calculated from the log portions of the curves are: for STS with agar, 5 hours 20 minutes; for STS with gelatin, 9 hours 15 minutes; for STS without any viscous substance, 9 hours 20 minutes; for STS with methyl cellulose, 6 hours; and for STS with mucin, 11 hours. Further examination of the curves reveals that the highest maximum counts of protozoa (about 3.3×10^6 organisms per ml) are found on STS with 100 mg percent agar. It is also on this medium that the flagel-

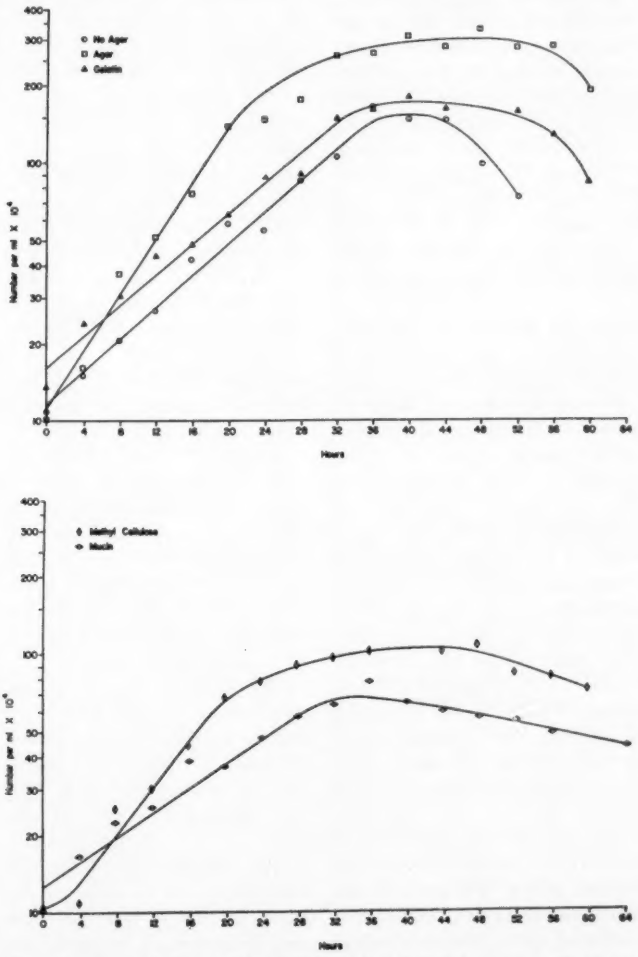

FIGURE 5. Growth curves of Jones' Barn (JB) strain of *Trichomonas gallinae* maintained on STS with various viscous substances and without any such substance ("no agar").

lates remain longest in the stationary phase. On STS with 800 mg percent gelatin the maximum counts are lower (about 1.8×10^6 organisms per ml) and the stationary phase a little shorter. While the maximum counts of trichomonads maintained on STS without any viscous substance (about 1.6×10^6 organisms per ml) approach those obtained on STS with gelatin, the stationary phase seems to be very short, lasting only about 8 hours; after 8 hours the cultures enter the phase of logarithmic death. Although the growth rate of the flagellates maintained on STS with 75 mg percent methyl cellulose approaches that observed on STS with agar, and although the cultures remain for an apparently very long time in the stationary phase, the maximum counts reached on this medium (about 1×10^6 organisms per ml) are much lower than those seen in JB strain grown on all of the previous media. Finally the trichomonads maintained on STS with 300 mg percent mucin not only exhibit the longest generation time and reach the lowest maximum numbers (about 8×10^5 organisms per ml), but also seem unable to remain in the plateau for more than a few hours.

In some respects the behavior of the parasites in the several experimental culture media corresponds to their behavior after injection in these media into the subcutaneous tissue of mice. There are obvious similarities in the generation times and the maximum numbers of the flagellates maintained on STS in gelatin and on STS without any viscous substance. It appears thus that gelatin has little influence on either the parasites or on the host tissues. The fact that the culture, when grown on STS without any viscous substances, reaches the phase of logarithmic death much sooner than when maintained on STS with gelatin may be easily explained by the slower rate of oxygen diffusion in the latter medium. While of some advantage to the parasites in vitro, this substance could not, for obvious reasons play any such role in the tissues of the experimental hosts. There is also little doubt that agar provides the most favorable conditions for the parasites both in vivo and in vitro. (It was ascertained that the flagellates multiplied fastest and reached the highest maximum numbers in the lesions produced in the presence of agar.) Although methyl cellulose seems to have some stimulatory effect on the initial division rate of the flagellates (this may depend in part at least on a decrease in the diffusion rate of oxygen in the medium), it appears to inhibit this rate in the later stages of development of the culture. Furthermore, it can be established that the flagellates present in the abscesses produced by inocula that contain this substance are neither as numerous nor as lively as those found in the lesions that are caused by injections of cultures with agar. These findings appear to emphasize that the main role played by methyl cellulose in enhancing the lesion-producing capacity of the parasites is that of an irritant and that the influence of agar is more than one of a simple irritant. At the present time, our knowledge of all the aspects of the role played by agar in trichomonad cultures is very limited and the same may be said in respect to our understanding of the influence which this substance may have, in addition to the irritation of the host tissues, on the metabolism and ultimately upon the virulence of the parasites to mice.

As far as mucin is concerned, it seems evident that it inhibits the growth of the flagellates when present in 0.3 percent concentration. On the other hand, it can be ascertained that the same substance in 0.1 percent concentration is stimulatory. In view of these findings it would not be at all surprising if the higher concentration of mucin affected detrimentally not only the flagellates in culture but also in the host tissues. Consequently the relatively limited effect which mucin had upon the formation of lesions might have been one of an irritant. It is entirely possible that in lower concentrations this substance, affecting the physiology of the flagellates, could enhance their virulence to mice to a much higher degree.

All the foregoing experiments give only a very limited insight into the role played by various environmental factors in enhancing the virulence of trichomonads to mice. They clearly emphasize the important, albeit not clearly understood part played by agar. It would be of great interest to learn which materials found in the natural hosts of the trichomonad parasites may exert an influence similar to that of agar. Bacteria or their metabolic products suggest themselves as the most likely counterparts.

DISCUSSION

There can be no doubt that *T. gallinae* contains a number of pathogenic strains which vary in their degree of virulence to both the natural and the experimental hosts. It appears equally certain that some of the strains are very mild and seem almost avirulent, to their natural hosts at least. As pointed out by Stabler (1948, 1954) in reference to the natural hosts and confirmed by many present experiments which employed experimental hosts, the basic degree of virulence of the various strains is an inherent physiological characteristic or depends upon a series of such attributes. While the degree of virulence can be affected by environmental conditions, a comparison of the relative pathogenicity of various strains under different but uniform sets of conditions always shows differences which may be ranked in the same order. The most pathogenic strain always remains so, and the same is true of those which exhibit the lowest or intermediate degrees of virulence. One of the inherent factors which seems to affect the degree of pathogenicity is the rate of multiplication of the parasites in the natural and experimental hosts. In reference to the strains of *T. gallinae* the rates of growth in culture reflect their rates in the hosts. It is clear, however, that the rate of growth constitutes only one of the factors influencing pathogenicity. Our experiments with tissue cultures (see Honigberg and McLure, 1960) and the work of others indicate the existence of many such factors.

While no parasitologist would deny the fact that *T. gallinae* contains truly pathogenic strains, there are those who, overwhelming evidence to the contrary notwithstanding (see discussion above), still appear to doubt the existence of true pathogens among the strains of *T. vaginalis*. They claim that it is "other" factors in the environment which, although they favor the flagellates, are the primary causes of the pathological changes and the accompanying symptoms. No one can question the important influence of environmental conditions upon the expression of virulence among parasites in general and trichomonad flagellates in particular. Still, the information obtained from experimental infections in the natural hosts (see Trussell, 1947) and in laboratory animals (see the summary of previous investigations and the original data in this report) indicates that among the strains of *T. vaginalis*, the potential pathogenicity is an expression of inherent physiological attributes. If there is no doubt that some of the strains of *T. gallinae* are bona fide pathogens, there would be none that the same is true of those of *T. vaginalis*. There is also a good deal of evidence that, as with *T. gallinae*, the degree of pathogenicity which the strains of the human genital trichomonad exhibit in mice closely resembles their relative virulence to the natural hosts.

The results reported in the present paper posed more questions than they answered. Some of these questions we hope will be answered in the continuation of this work or by others.

SUMMARY

A survey of literature dealing with inoculations of various strains of *Trichomonas gallinae* (Rivolta) and *T. vaginalis* Donné to mice is followed by an account of pathogenicity to the natural hosts of the strains of these two species which were employed in the present investigation.

The growth curves and generation times of the several strains on BBL fluid thioglycollate with 5 percent normal horse serum are compared. It is evident that among the strains of the avian species there is a good correlation between rate of growth in vitro with that in mice, and also with the relative pathogenicity of the strains to these experimental hosts. On the other hand, it appears that the more virulent the strains of *T. vaginalis*, the longer their generation times in vitro. Still, the rates of growth exhibited by these strains in vivo show a positive correlation with their degree of pathogenicity.

The relative susceptibility of mice to trichomonads was demonstrated by four successful serial passages of Jones' Barn strain of *T. gallinae* in 6- to 8-week-old C57 B1/6 mice, which were employed in all experiments reported in this paper. The gross pathology observed in mice after subcutaneous inoculations of four strains of *T. gallinae* and four of *T. vaginalis* is discussed, and the morbid changes found in the present investigation are compared with those reported by various investigators who employed other strains of both species.

A statistical comparison of the mean volumes of subcutaneous lesions produced in mice by the several strains of both species reveals that these volumes, which express the degree of

pathogenicity of the strains to the experimental hosts, faithfully reflect their relative virulence to the natural hosts. This correlation can be established with a greater degree of certainty in respect to the strains of *T. gallinae* than in reference to those of *T. vaginalis*. However, there is much evidence that it holds true also in the latter instance. Furthermore, the data indicate that the least virulent strains of the human genital trichomonad are still more harmful to mice than the least pathogenic strains of the avian species.

It has been found that strains of both species become attenuated in their virulence when maintained in culture. This attenuation appears more pronounced in *T. gallinae* than in *T. vaginalis*.

The presence of agar in the cultures of strains of both trichomonad species and in the inocula enhances the development of subcutaneous lesions. There is some experimental evidence indicating that the effects of agar are not confined to its action as an irritant upon the host tissues, but that this substance must have some influence upon the physiology of the parasites rendering them more pathogenic. Although methyl cellulose when substituted for agar in the culture medium enhances the virulence of the parasites to a less degree than agar, the abscesses produced in the presence of the former are still quite large. Smaller lesions result from inocula that contain mucin in place of agar. Abscesses produced by the parasites in the presence of gelatin are no larger than those caused by inoculations of trichomonads grown on media which include no viscous substances. The possible role played by the several substances is discussed in the light of their physicochemical properties and their influence upon the parasites and the host tissues.

Our data indicate that although environmental conditions play an important role in modifying pathogenicity, the basic degree of virulence is an expression of physiological characteristics inherent in the strains of both *T. gallinae* and *T. vaginalis*. They also establish the fact that *T. vaginalis* contains truly pathogenic strains.

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PLATE I

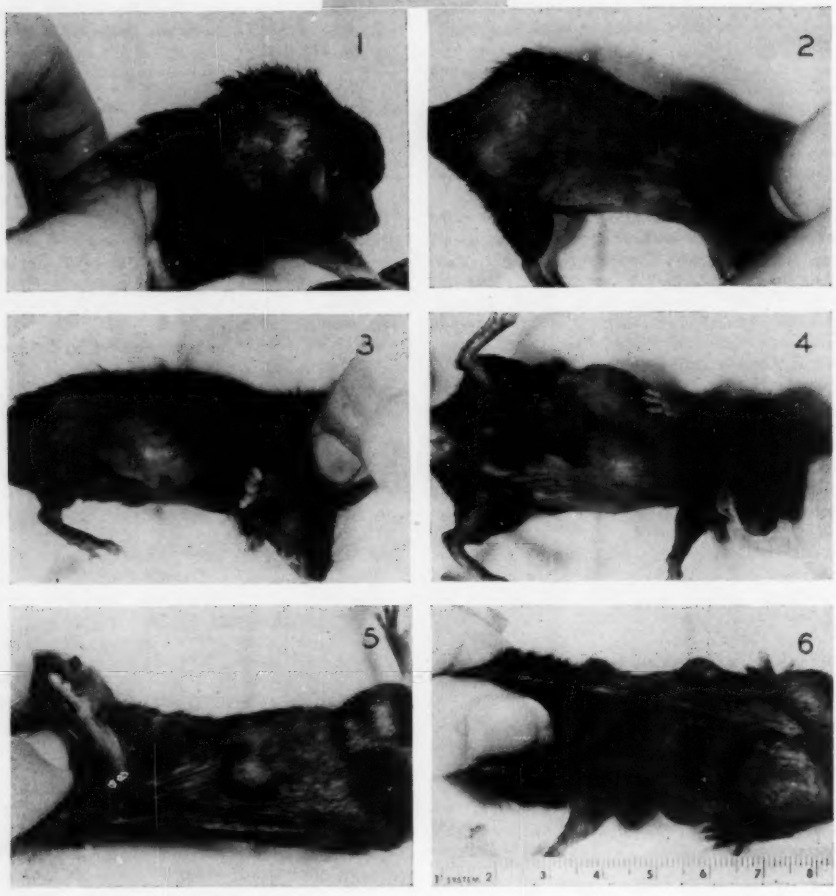

PLATE I

All photographs represent 14-day lesions caused by subcutaneous inoculations of two different strains of *Trichomonas gallinae*. Figures 1 to 4, Jones' Barn (JB) strain; figures 5 and 6, Lahore (YG) strain.

FIGURE 1. Gas-filled spreading type of lesion; entire skin surface of animal is spongy and exhibits pronounced edema.

FIGURE 2. Type of lesion similar to that shown in figure 1; note however that edema is far less generalized.

FIGUER 3. Top view of large hard abscess.

FIGURE 4. Side view of abscess shown in figure 3.

FIGURE 5. Top view of small hard abscess.

FIGURE 6. Side view of abscess shown in figure 5.

PLATE II

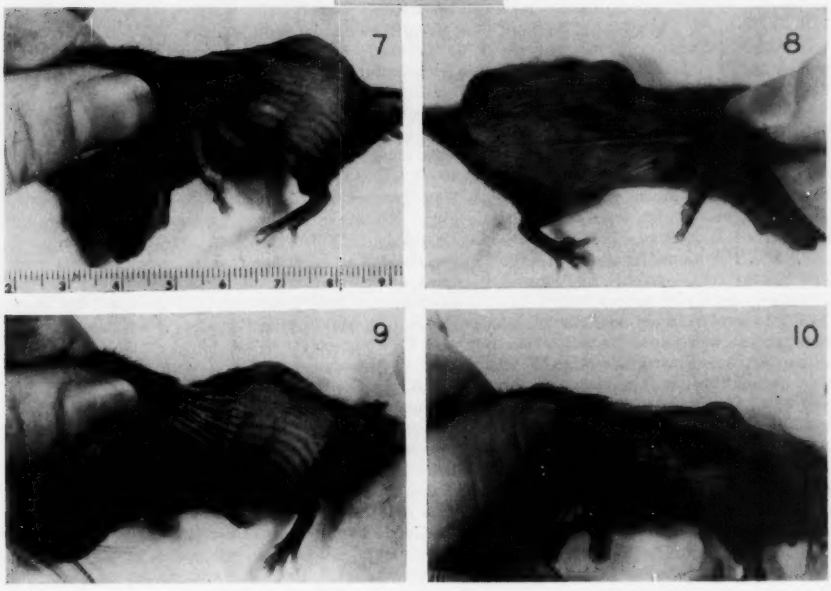

PLATE II

All photographs represent 14-day lesions caused by subcutaneous inoculations of two different strains of *Trichomonas vaginalis*. Figures 7 and 8, Cavanagh strain (TVC); figures 9 and 10, strain No. 1 (TV 1)

FIGURE 7. Top view of medium-size hard abscess.
FIGURE 8. Side view of lesion shown in figure 7.
FIGURE 9. Top view of small hard abscess.
FIGURE 10. Side view of lesion shown in figure 9.

RESEARCH NOTES

ATTEMPTS AT INTERSPECIES GENETIC TRANSFER IN TRYPANOSOMES

Ever since the work of Avery, MacLeoud and McCarty (1944, J. Exp. Med. **79**: 137–157) there has been considerable interest in genetic transfer via nucleic acid macromolecules into the genome of a recipient cell. Successful genetic transformation has been accomplished since on many occasions among the bacteria. In addition, Fulton (1960, Rutgers Biol. Symp. Host. Infl. on Parasite Physiol., pp. 11–23), Honigberg and Read (1960, Science **131**: 352–354) and Inoki and Matsushira (1960, Biken's J. **3**: 101–106) have reported on what appears to be nucleic acid mediated intra species genetic transfer among trypanosome and trichomonad flagellates.

The present note reports on attempts to effect nucleic acid mediated genetic transformation between two different species of trypanosomes, i.e., data on inter species transformation. The Tulahuen strain of *Schizotrypanum cruzi* was mass-cultured in a modified Chang's diphasic medium (Chang, 1947, J. Inf. Dis. **80**: 164–171). After centrifugation and washing with buffered saline, culture forms of *S. cruzi* were lysed by addition of deoxycholate, ultrasonic disintegration, or by repeated freeze-thawing. Nucleic acids were extracted following the methods of Hotchkiss (1957, Methods in Enzymology, Vol. III, Acad. Press). For preparations of DNA alone, RNAse was added to remove RNA. In either case the nucleic acids were precipitated with ethanol and stored in small quantities of saline. Blood stream forms of *Trypanosoma lewisi* were obtained by cardiac puncture from laboratory rats having been given *T. lewisi* by i.p. injection 4 to 10 days previously. A small quantity of *S. cruzi* nucleic acid preparation was added to either the citrated *T. lewisi*-infected rat blood or to a buffered saline Armour Plasma Fraction V medium to which washed *T. lewisi* were added. The live *T. lewisi* cultures with nucleic acid from *S. cruzi* were kept in vitro at temperatures of 25, 30, and 37 C, respectively, for various lengths of time up to 12 hours. Then this material, containing live *T. lewisi*, was injected i.p. into C_3H mice and also fed through membranes to trypanosome-free nymphs and adults of *Triatoma protracta* which are suitable hosts for *S. cruzi*.

Since normally, *T. lewisi* produces no infection in either mice or triatomid bugs, the appearance of trypanosomes in mouse blood, leishmania forms in mouse heart muscle, or leptomonad and crithidial forms in the reduviid host after a suitable period of time, 10 to 20 days, would have been evidence of nucleic acid mediated transfer of infectivity from *S. cruzi* to *T. lewisi*. The results obtained from ten experiments comprising utilization of six separate nucleic acid preparations, and involving 48 mice and over 100 *Triatoma protracta*, failed to show any successful nucleic acid mediated transfer of infectivity from *S. cruzi* to *T. lewisi*.

(Supported by research grant E 2915 from the U. S. Public Health Service; technical assistance of C. D. Wiens and H. Speer is gratefully acknowledged.)—YOST U. AMREIN, *Department of Zoology, Pomona College, Claremont, California.*

THE OCCURRENCE OF MICROFILARIAE OF *ONCHOCERCA VOLVULUS* IN URINE OF INFECTED INDIVIDUALS.

Microfilariae of *Onchocerca volvulus* in man are usually confined to the skin, subcutaneous tissues, and eyes of infected individuals. In a few instances they have been reported from other locations in the body.

Recently during the course of studies on parasitic infections of Africans, urine samples were taken on over 300 individuals. On seven separate occasions, microfilariae of *O. volvulus* were found in the urine sediment. In the Orientale Province of the Congo, they appeared in the urine of two Pygmies from Vieux Kilo and four from Ofay. In East Africa, they were found in the urine of one individual from Nyabirongo in the Toro District of Uganda.

Since these microfilariae do not generally utilize the blood stream, the finding of microfilariae in the urine indicates that they migrate fore extensively through the tissues than has been previously reported. In addition, the author has for some time suspected that the adults of *O. volvulus*, like many other filarioid parasites, migrate within the intermuscular fascia rather than confine their movements to the subcutaneous tissues. The presence of microfilariae in the urine may be a result of the location of adults in the deeper tissues of the pelvic region.

Investigations to further advance the information on the activity of *O. volvulus* within the body are currently underway.—DONALD L. PRICE, *Walter Reed Army Institute of Research, Washington, D. C., and Makerere College Medical School, Department of Pathology, P. O. Box 2072, Kampala, Uganda, East Africa.*

EFFECT OF LOW TEMPERATURES ON TROPHOZOITES OF *GIARDIA MURIS*[*]

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In recent years, due to the difficulty often encountered in the propagation of parasitic protozoa, several individuals investigated the effects of low temperatures on these organisms; they hoped to utilize such techniques as a means of prolonged preservation of the living organisms. The first successful experiments were done by Coggeshall (1939). Manwell (1943) and other workers made additional contributions to the low temperature work on malarial parasites.

Polge, Smith and Parkes (1949) discovered that spermatozoa would remain alive and viable after storage at – 76 C, if the semen was stored in 10 percent glycerol. This discovery soon led to some attempts to apply this technique to various protozoans. Fulton and Smith (1953) found that *Entamoeba histolytica* trophozites survived at – 79 C after slow cooling in buffered horse serum —Ringers medium containing either 5 or 10 percent glycerol, for periods of 7 and 50 days, respectively.

Levine and his co-workers have recently reported some interesting work on the survival of *Trichomonas foetus* in glycerol. Levine and Marquardt (1954) reported the survival of this parasite at freezing temperatures. Joyner (1954) reported no survival of this same parasite in glycerol-treated semen. Levine and Marquardt (1955) cultured *T. foetus* in CPLM medium (Johnson and Trussell 1943) to which was added 5 or 10 percent glycerol. This mixture was then frozen at temperatures of – 20 C or – 76 C. They froze the parasites slowly in some experiments and rapidly in others. Their results showed that *T. foetus,* if frozen slowly, would survive freezing for periods up to 64 days. They did not survive quick freezing. They also conducted experiments in which they substituted compounds related to glycerol for the glycerol. None of the compounds utilized were as effective as glycerol.

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MATERIALS AND METHODS

The experimental techniques used in these experiments were similar to those employed by Levine and his co-workers. The flagellate studied was *Giardia muris* from the small intestines of mice. The initial difficulty encountered was due to the fact that there was no known medium which would grow and maintain any species of *Giardia.* A survival medium, which had previously been devised by the author, and in which the trophozoites remain alive and motile for approximately 3 days, was employed. This medium was composed of the following: Tyrode's solution, 50 ml; ascorbic acid, 0.02 g; L-cysteine HCl, 0.1 g; and Pangestin, a Difco preparation of pancreatic enzymes, 0.05 g. Tween 80, a surface active agent, was sometimes added to the Tyrode's as 1 percent of this solution. The pH was adjusted to 7.2 with NaOH. Glycerol was added in amounts ranging from 11 to 50 percent. Ethylene glycol was substituted for glycerol in one experiment.

The trophozoites used in this series of experiments were collected directly from the intestine of a heavily infected mouse. No attempt was made to sterilize the inoculum. The contents of the small intestine were mixed with the survival medium and the trophozoites checked for motility. The results were calculated on the basis of 100 percent motility. Motility of the flagella was used as the criterion for determining if the parasites survived freezing. The first 100 trophozoites, or the total number per slide if less than 100 were present, were checked for motility and the percentage of organisms living determined from this information.

The trophozoites, after collection from the host and mixing with the media, were placed in either flat-bottomed 22-by 122-mm or round-bottomed 16- by 125-mm screw-capped vials and mixed with definite quantities of glycerol. These tubes were then subjected to low temperatures. Several temperatures were used: (1) The first temperature was 8 C; (2) the second, – 20 C; (3) the majority of the tubes were stored at – 38 C ± 5 C, either wrapped in cotton or placed in the freezer without insulation; (4) a few tubes were stored at – 70 C. All of these were quick-frozen in a dry ice-alcohol bath.

When the tubes were removed from the cold for examination, they were allowed to stand at room temperature for approximately 1 hour. After this interval the medium warmed and the trophozoites were motile. The tubes were then sampled and checked, as previously described, to determine the number of *Giardia* alive. If a considerable number of living trophozoites were noted in a particu-

lar tube, the tube was sometimes refrozen to see if the parasite could tolerate this procedure.

RESULTS

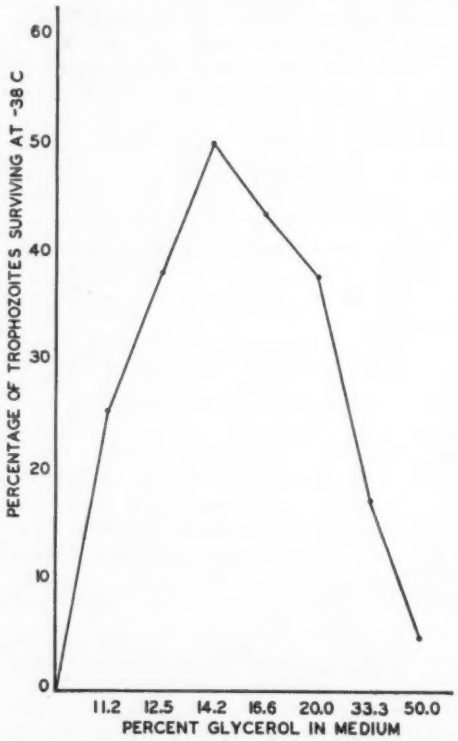
The results of this experiment indicated that *G. muris* and *T. foetus* react to cold in the presence of glycerol in a somewhat similar manner. No living trophozoites were observed after 24 hours exposure at 8 C. At – 20 C, – 38 C and – 70 C the parasites would survive at least 24 hours if glycerol were employed in the medium. A total of 118 control tubes, without glycerol, were checked throughout the course of the experiments. No living or dead trophozoites, at any of the temperatures employed, were ever observed in the control tubes. The cells had lysed and disappeared after 24 hours. A total of 330 tubes containing glycerol were examined. One hundred and thirty-eight or 41.8 percent of these tubes contained living *Giardia* trophozoites.

At – 20 C, 18 tubes were used containing 33.3 percent glycerol and 18 with 50 percent glycerol. The results showed a 15.3 percent average survival per 100 organisms per tube containing 33.3 percent glycerol and no survival in the tubes containing 50 percent glycerol.

Many more samples were exposed to – 38 C than – 20 C. This temperature appeared, in some of the initial work, to be the most favorable for the survival of *G. muris*. Utilizing this temperature, additional factors that might influence the survival of *Giardia muris* in the cold were examined. Figure 1 shows the effect of various glycerol concentrations on the survival of the trophozoites at – 38 C. A concentration of 14.2 percent glycerol gave the best results. As the concentration was increased or decreased the number of living organisms present decreased. The rate of cooling and the presence of Tween 80 in the medium also appeared to influence the survival of *G. muris* at – 38 C.

Slow freezing in cotton insulated tubes resulted in a higher survival rate than did quick freezing, though the survival rate is low under both conditions. These results agree in general with the results obtained by Levine and Marquardt (1955) with the exception that they reported no survival of *T. foetus* after thawing and refreezing. It was also noted that if the basal medium contained 1 percent by volume of Tween 80 and glycerol was added, the number of living trophozoites present after freezing and thawing was greater than when the Tween 80 was not present in the medium.

A few tubes of inoculated medium containing 5 different concentrations of glycerol were exposed to – 70 C. The samples were cooled rapidly to freezing by immersing the tubes in a dry ice-alcohol bath. All of the tubes contained Tween 80. There was no survival at the higher concentrations of 80, 66.6, and 50 percent glycerol. In lower concentrations of 33.3 and 20 percent glycerol, survival was good. The extremely rapid freezing of the tubes at these temperatures did not kill the trophozoites. In fact the limited number of tubes used, four in each case, showed an average of over 50 percent survival, 53 percent for the tubes with 33.3 percent glycerol, and 69 percent for the tubes containing 20 percent glycerol. As only a small number of tubes were used, these figures may be high, but the results do show that the trophozoites of *G. muris* will survive quick freezing at a low temperature.



An attempt was made to substitute ethylene glycol for the glycerol. There was no survival in any of the 20 tubes containing concentrations of ethylene glycol ranging from 10 to 50 per-

cent.

Several tubes were also checked to determine if a normal saline solution could be substituted for the basal survival medium. Survival in normal saline was zero; in Tyrode's solution, which is actually a part of the basal survival medium, the average survival was 5.1 percent.

In all experiments, where fairly large numbers of tubes were examined under the same conditions, a tremendous variation was noted in the number of living organisms. The range was from 0 to 97 percent survival. Levine (1959) noted this same situation in his work with *T. foetus*.

The length of time *G. muris* will survive at low temperatures has not been determined past 27 days for unthawed samples and 40 days for refrozen samples. The number of tubes examined was small and the number of surviving organisms was also small, but *G. muris* will survive prolonged exposure to low temperatures.

DISCUSSION

An explanation of the action of glycerol was proposed by Lovelock (1953, 1954) from information derived from work on hemolysis of red blood cells. He stated that glycerol functions as a "salt buffer." If the temperature is lowered, more water is withdrawn and the salt concentration of the cell increases. If glycerol is added to the salt solution, the concentration of salt in equilibrium with ice is lowered for any temperature during freezing or thawing. As a result the cells remain intact in the presence of glycerol, while without it the cells will lyse.

Lovelock (1954a) listed four characteristics that a substance should possess in order to protect cells during freezing and thawing: (1) Low molecular weight, (2) miscibility with salt solutions, (3) non-toxicity, and (4) ability to permeate the cell freely. A surface activating agent such as Tween 80 could increase the permeability of the cell wall and thus increase the protective ability of glycerol for cells exposed to subzero temperatures.

The concentration of glycerol used should be approximately 14 percent of the total volume of the medium for maximum efficiency when freezing *G. muris*. Increasing or decreasing the concentration decreases its ability to protect the trophozoites at low temperatures.

In all of the tubes checked for trophozoites no cysts were ever seen. The trophozoites either persisted in that form or it lysed completely. Cyst formation apparently is not dependent upon an adverse temperature situation. Temperature may play no part in cyst formation.

Insulation of the tubes at the time they are frozen aids in the survival of the parasites. *G. muris* will, however, survive quick freezing in a dry ice-alcohol bath. The tubes were, in this case, allowed to stand for about 30 minutes before immersion in the dry ice-alcohol bath to allow time for the glycerol to penetrate the cell.

SUMMARY

Giardia muris can survive cooling to low temperatures and subsequent thawing in the presence of glycerol. Retarding the rate of freezing by utilizing cotton as the insulating material appears to facilitate the survival of *G. muris* at −38 C, as does the addition of Tween 80 to the medium. The trophozoites will survive quick freezing at −70 C. The parasite can survive in the frozen state for 40 days and can survive thawing and refreezing.

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RESEARCH NOTE

BESNOITIA FROM AN OPOSSUM

Members of the genus *Besnoitia* are highly pathogenic, *Toxoplasma*-like organisms. The extreme similarity of their spores to those of *Toxoplasma* was pointed out by Goldman et al (1957, J. Parasit. **43**: 490–491). Two species have been described: *B. besnoiti,* from Old World cattle and horses; *B. jellisoni,* originally reported from a deer mouse, *Peromyscus maniculatus.* Thorough discussions of these species have been presented (Frenkel, 1955, Atti VI Cong. Internaz. Microbiol. Roma 6–12 Sett. 1953, 5: 426–434; Jellison, 1956, Ann. N. Y. Acad. Sci. **64**: 268–270; Jellison et al, 1956, Ann. N. Y. Acad. Sci. **64**: 271–274; Jellison et al, 1960, Bol. Chileno Parasit. **15**: 46–47). The recent finding of *Besnoitia* by one of us (KW) in a new host, the opossum, is the basis for this note.

The host was an adult opossum (*Didelphis marsupialis*) captured near Paris, Texas. An examination of the cranial dural venous sinuses revealed two yellow-gray masses. One was in the lateral sinus, the other in the inferior petrosal sinus. Microscopic examination proved them to be the cysts of *Besnoitia.* They measured 548 by 685 microns and 514 by 754 microns, respectively. They contained the usual vast numbers of banana-shaped spores. The cystic membranes were quite obvious and bore the scattered, large nuclei typical of members of the genus.

Since no reason for describing a new species exists, the present cysts are being identified as those of *Besnoitia jellisoni* Frenkel, 1955. Aside from the numerous laboratory animals into which it has been successfully placed, this species has been found occurring naturally in the original host, a *Peromyscus maniculatus* from Idaho (see Frenkel, 1955), in three of seven *Microxus torques* from Peru (see Jellison et al, 1960), and in one *Dipodomys* sp. from Utah (Jellison, 1960 per. comm.). All the recorded hosts of *B. jellisoni,* therefore, have been rodents. The present record is of interest in that the opossum represents a new type of host, a marsupial. Moreover, it has been shown that transmission is possible through cannibalism involving infected mice (see Jellison et al, 1956). As opossums are quite willing to consume these rodents, mice might have constituted the source of infection for the present host.

We are grateful to Dr. J. K. Frenkel of the University of Kansas Medical Center and to Dr. W. L. Jellison of the Rocky Mountain Laboratory for confirming the diagnosis and for helpful suggestions. ROBERT M. STABLER, *Colorado College, Colorado Springs,* AND KEASLEY WELCH, *University of Colorado Medical Center, Denver.*

AN ELECTRON MICROSCOPIC STUDY OF *ENTAMOEBA HISTOLYTICA**

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Osada (1959) published an electron microscope study of *Entamoeba histolytica*. His description of the fine structure was incomplete and based solely on osmium tetroxide fixation.

Luft (1956) using potassium permanganate and Low and Freeman (1959) using chromium compounds as fixing agents have demonstrated their usefulness for the preservation of certain fine cellular structures. These techniques and others were used to authenticate structure in *E. histolytica* as seen with osmium tetroxide fixation.

MATERIALS AND METHODS

Trophozoites of *E. histolytica* (NRS strain) were grown in a modified Boeck-Drbohlav medium consisting of saline overlaid egg slants with rice starch and a varied bacterial flora. Trophozoites from 48-hour culture were fixed according to the following procedures: (1) 30 minutes in 1 percent osmium tetroxide buffered with veronal to a pH of 7.4; (2) 15 minutes in a 3 percent chromic oxide, 4 percent formalin and 0.85 percent sodium chloride solution as recommended by Low and Freeman (1956); (3) 15 minutes in a 3 percent chromic oxide solution; (4) 15 to 30 minutes in 0.6 percent potassium permanganate buffered with veronal to a pH of 7.4 as recommended by Luft (1956). Trophozoites were fixed at a temperature of 0 to 5 C. Material was dehydrated in a graded series of methyl alcohols with occasional gentle centrifugation and embedded in a 3:1 mixture of *n*-butyl and methyl methacrylate. Polymerization was carried out at 45 to 50 C. Thin sections were cut on a Porter-Blum microtome, mounted on formvar covered grids and examined in a RCA EMU-3 at 50 KV.

OBSERVATIONS

Cytoplasm

The plasma membrane may be smooth or very irregular in appearance according to the activity of the ameba at the time of fixation. The cytoplasm contains vacuoles of various sizes which may be empty or contain rice starch and partly digested bacteria. The membranes of these vacuoles appear identical with the plasma membrane.

Osmium-fixed specimens show no differentiation of endoplasm and ectoplasm. However, with potassium permanganate fixation areas of electron density are seen within the plasma membrane (figs. 2 and 3). Only scattered densities are seen in what would correspond to the endoplasm.

Neither Golgi apparati nor mitochondria were seen in sections.

The cytoplasm exhibits fine alveolation with scattered circular and less often oval profiles corresponding to an endoplasmic reticulum of limited organization. A few dense particles are usually found at the periphery of the profiles.

Nucleus

The nucleoplasm has a greater electron density than the cytoplasm. It is more granular in nature than the finely alveolated cytoplasm.

The nuclear membrane *E. histolytica* cannot be as clearly visualized with osmium or other fixatives as those of metazoan cells or free-living protozoa. However, in some sections it is clearly double with inner and outer elements separated by a narow area of low density (fig. 9). There are numerous discontinuities in the membrane with osmium fixation. Various fixation times yielded the same results. Therefore, it seems likely that these discontinuities represent pores in the membrane. The interstices of such a pore system may compose as much as 50 percent of the area of the nuclear membrane. Pores are usually associated with low density areas within the nuclear membrane (figs. 4 and 5).

Large irregularly shaped electron densities, composed of fine particles, are located at the periphery of the nucleus. These correspond to the "beaded" areas within the nuclear membrane

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* Supported in part by grants E-2347 and H-2549, National Institutes of Health, U. S. Public Health Service.

of *E. histolytica* in light microscopy. In the region of nuclear pores this material is not in contact with the nuclear membrane (figs. 4 and 5).

The karyosome of *E. histolytica* is composed of a number of dense irregular areas made up of particles with the same size range as the granules of the peripheral "beaded" area (figs. 1, 4 and 6). The karyosome is not observed in electron micrographs to be the single body usually observed in light microscopy.

Certain discrete structures are usually observed at the periphery of the nucleus with osmium fixation. These nuclear structures are aggregates of granules most of which measure about 100 A (angstrom). They are larger than the granules of the "beaded" areas and, although located among the latter, are limited to a much smaller area of the nucleus (figs. 1, 4, 5 and 6). In successive sections the aggregates may branch or join with others in the same area. Occasionally the structures appear as a ring of granules encircling a space, which may or may not contain other granules, or there may be concentric rings of granules (fig. 8). Rarely are the granules located in the area of the karyosome or at the periphery of the nucleus (figs. 7 and 8). However, when this is observed the granules are large perhaps due to coalescense as they frequently are paired or in the form of triads and tetrads.

Chromic acid-formalin fixation causes these structures to lose some of their granular nature and assume a more filamentous organization of greater electron density than obtained with osmium fixation (figs. 10 and 11).

Potassium permanganate fixation shows them as granular aggregates of great density (fig. 12).

The fixation of trophozoites with 3 percent chromic oxide completely destroys the nucleoplasm and nuclear membrane. However, the chromatin plaques and karyosome remain intact, although their granular nature is destroyed. Small dense bodies with a somewhat filamentous organization, characteristic of the bodies seen with chromic acid-formalin fixation, are present among the chromatin plaques (fig. 13).

DISCUSSION

Permanganate fixation demonstrates electron dense areas more commonly just within the cell membrane than deeper in the cytoplasm. Luft (1956) has shown that the permanganate ion enhances tissue density and contrast and reduces certain lipoprotein complexes. Therefore, since electron dense areas are more common just within the cell membrane, they may represent a different concentration of lipoprotein between ectoplasm and endoplasm.

Deutsch and Zaman (1959) found that mitochondria were lacking in *E. invadens*, and postulated that oxidative enzyme systems might be lacking or scattered in the cytoplasm. We found no evidence of mitochondria in *E. histolytica*. Osada (1959) and Deutsch and Zaman (1959) were also unable to demonstrate mitochondria by electron microscopy.

Osada (1959) described the pore complex of the nuclear membrane of *E. histolytica* as "several minute holes." The micrographs in our study suggest a much greater porosity. Porter (1957) and Swift (1958) have shown that pores in the nuclear membrane of various tissues are usually associated with interchromatin areas within the nucleus. In our study discontinuities in the nuclear membrane are invariably associated with low density areas in the peripheral "beaded" areas. The identification of these "beaded" areas as chromatin material, however, still remains in doubt.

Deutsch and Zaman (1959) have noted small "differentiated areas" just within the nuclear membrane of *E. invadens*. These areas are described as "a number of minute spaces encircled by very dark granules measuring about 100 A." Osada (1959) described chromatin granules in *E. histolytica* as "situated close to the nuclear membrane or sometimes at the center of the nucleus. Each of these chromatin granules has a tiny clear spot at the center."

These "chromatin granules" are identical to the granular aggregates we have observed in the chromatin material of *E. histolytica*. The "differentiated areas" in *E. invadens* are similar if not identical to the same structures in *E. histolytica*. The possibility that these granular aggregates are fixation artifacts may be discounted since they are present with various fixatives. On the basis of their preservation with permanganate they may be a lipoprotein complex.

SUMMARY

1. Trophozoites of *E. histolytica* (NRS strain) from 48-hour cultures were fixed with

osmium, chromium compounds, and potassium permanganate, sectioned, and examined.

2. An account of the fine structure is given. Some of the unusual features of this organization are the lack of the Golgi apparatus or mitochondria, the rudimentary endoplasmic reticulum, and the pore system of the nuclear membrane.

3. Granular aggregates in the peripheral "beaded" areas of the nucleus were observed with all of the fixatives employed and represent valid structures rather than fixation artifacts.

ACKNOWLEDGMENT

The authors wish to thank Dr. Robert L. Simmons, Department of Public Health and Preventive Medicine, who prepared the photographic prints for this publication.

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EXPLANATION OF PLATES
Entamoeba histolytica

PLATE I

FIGURE 1. OsO_4, 18,700 ×. Section of trophozoite showing nucleus. Many food vacuoles of various sizes present (V), some containing partly digested bacteria; cytoplasm exhibiting fine alveolation with scattered circular and less often oval profiles of an endoplasmic reticulum; small particles of varying density and size singly and in aggregates scattered through the cytoplasm; mitochondria and Golgi material not observed. The nucleus (N) contains a visible karyosome (K) and peripheral densities representing the "beaded" area of light microscopy (C). Small dark areas at the periphery of the nucleus are the granular aggregates (arrows).

PLATE II

FIGURE 2. $KMnO_4$ 21,250 ×. Portion of trophozoite demonstrating areas of electron density at the plasma membrane (arrow).

FIGURE 3. $KMnO_4$ 18,000 ×. Portion of a trophozoite with a large electron dense area at the plasma membrane (arrows); vacuoles (V) are more numerous in this region (see also fig. 2); some scattered densities also visible at some distance from the plasma membrane.

PLATE III

FIGURE 4. OsO_4 37,500 ×. Section through the nucleus of a trophozoite; a nuclear pore system represented by interstices in the nuclear membrane associated with low density areas in the peripheral "beads;" a few shown by arrows (see also fig. 5). Plaques of chromatin material, portions of the karyosome (K), and granular aggregates (GA) shown.

FIGURE 5. OsO_4 45,940 ×. Section through the nucleus of a trophozoite, nuclear pores clearly seen; granular aggregates (GA) easily differentiated from the material of the peripheral "beads."

PLATE IV

FIGURE 6. OsO_4 32,800 ×. Thick section through nucleus of trophozoite; karyosome (K) visible as a number of electron dense areas; dense material (the peripheral "beads") observed lining the nuclear membrane. Certain discrete rounded bodies, the granular aggregates (arrows), are indicated.

FIGURE 7. OsO_4 30,470 ×. Section through nucleus of a trophozoite; dense particles of various sizes seen in the region of the karyosome (K); these particles rarely seen in this position and then only when granular aggregates (figs. 4 and 5) or large particles (fig. 9) are not found

FIGURE 8. OsO_4 22,500 ×. Section through nucleus of trophozoite; granular aggregates appearing as single or concentric rings of granules (arrows) in this nucleus, a configuration infrein the peripheral "beads."
quently observed.

PLATE V

FIGURE 9. OsO_4 63,280 × (insert 9,375 ×). Section through nucleus of trophozoite; enlargement of areas of the insert (brackets) showing a double nuclear membrane (arrows) and large particles scattered throughout the peripheral "beads," these particles composed of smaller granules associated as pairs, triads or tetrads. The scattered particles are seldom seen, but when observed the granular aggregates are not found.

PLATE VI

FIGURES 10 and 11. CrO_3 46,875 ×. Two sections through nucleus of different trophozoites; nuclear membrane and peripheral "beads" poorly preserved with this fixative; however, granular aggregates (GA) usually deeply stained (fig. 10) somewhat filamentous (fig. 11) and perhaps appearing as concentric rings.

PLATE VII

FIGURE 12. $KMnO_4$ 52,500 ×. Section of portion of nucleus of trophozoite; some of the granular aggregates (GA) become extremely dense with this fixation. The lightly stained granular aggregates appear less filamentous than with CrO_3 fixation (figs. 10 and 11).

FIGURE 13. CrO_3, 3 percent 28,125 ×. Section through nucleus of trophozoites. (Fixative has caused severe damage to nucleoplasm and nuclear membrane; however, the peripheral "beads" and karyosome (K) remain intact, although altered in structure.) Small dense bodies (arrows) located in the peripheral "beads" represent the granular aggregates seen with other fixatives.

PLATE I

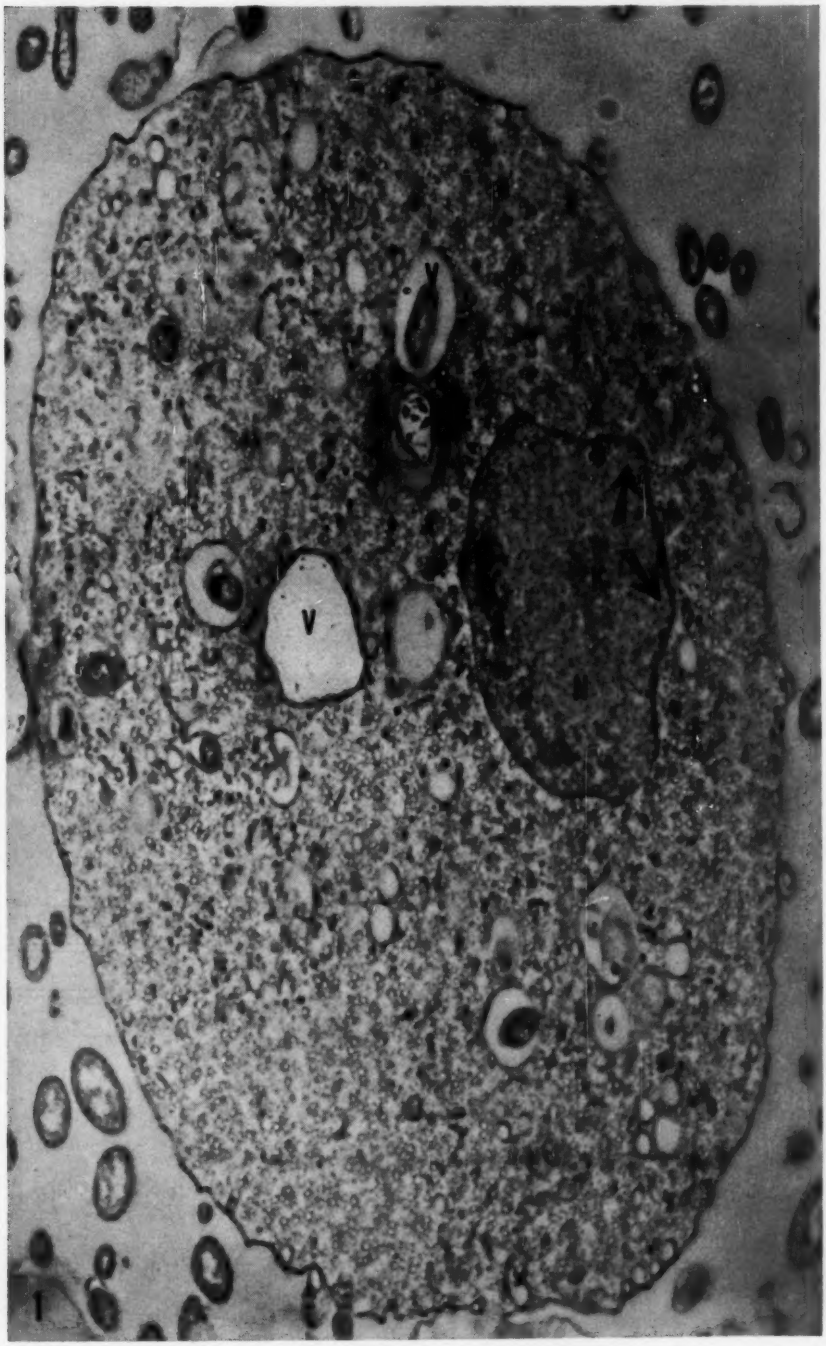
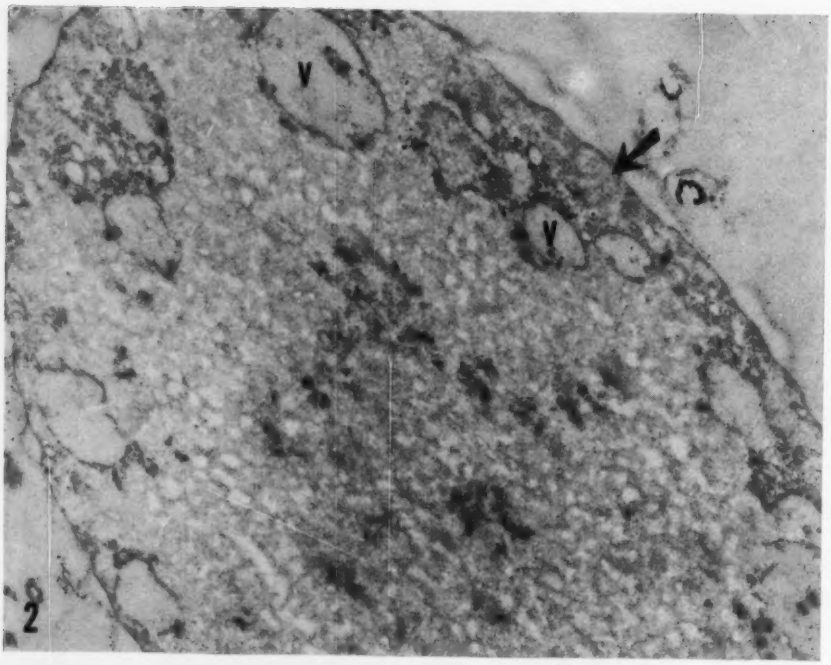


PLATE II



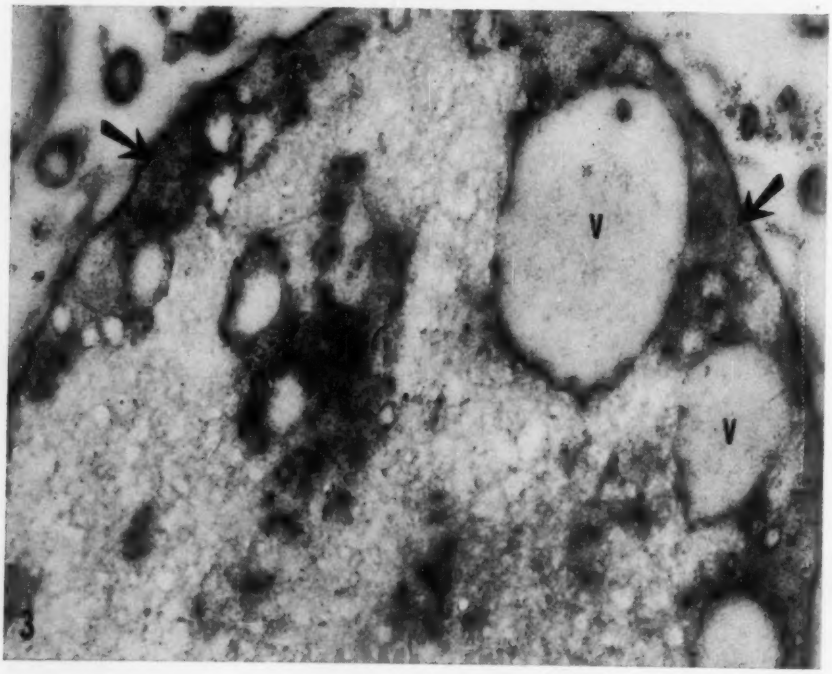
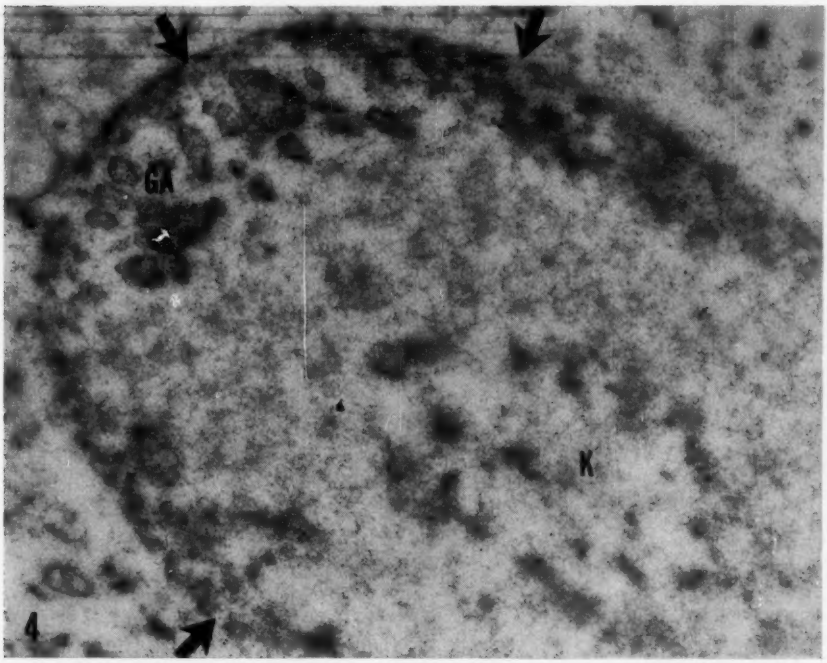


PLATE III



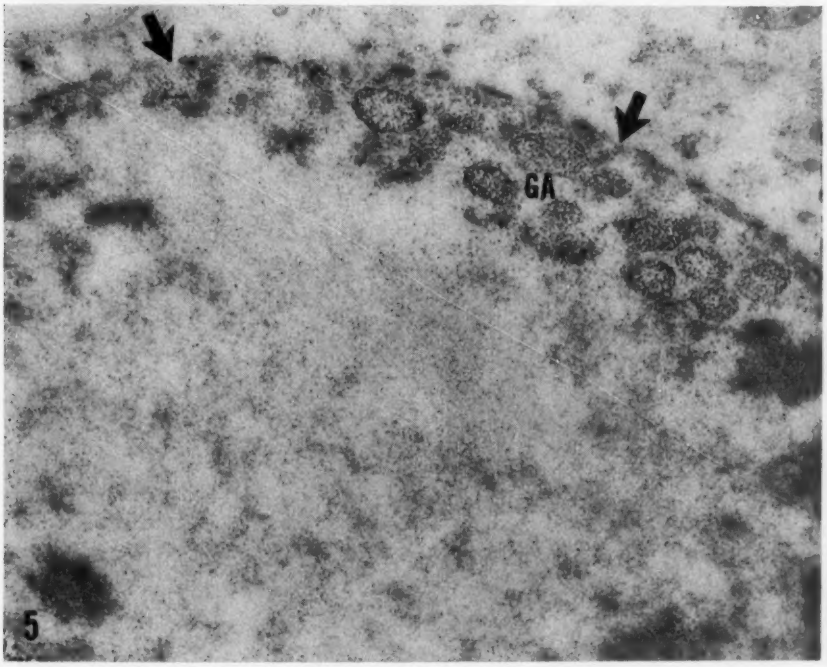
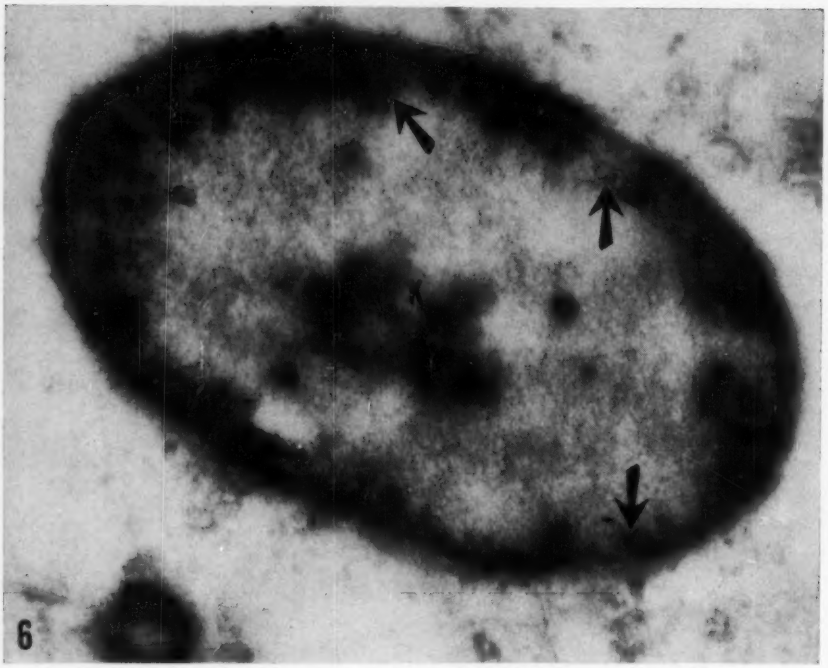


PLATE IV



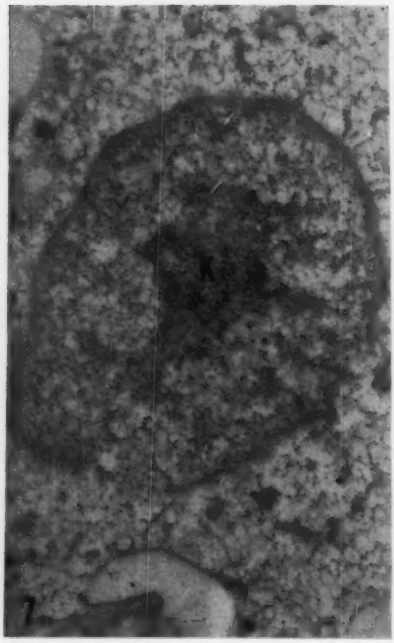


PLATE V

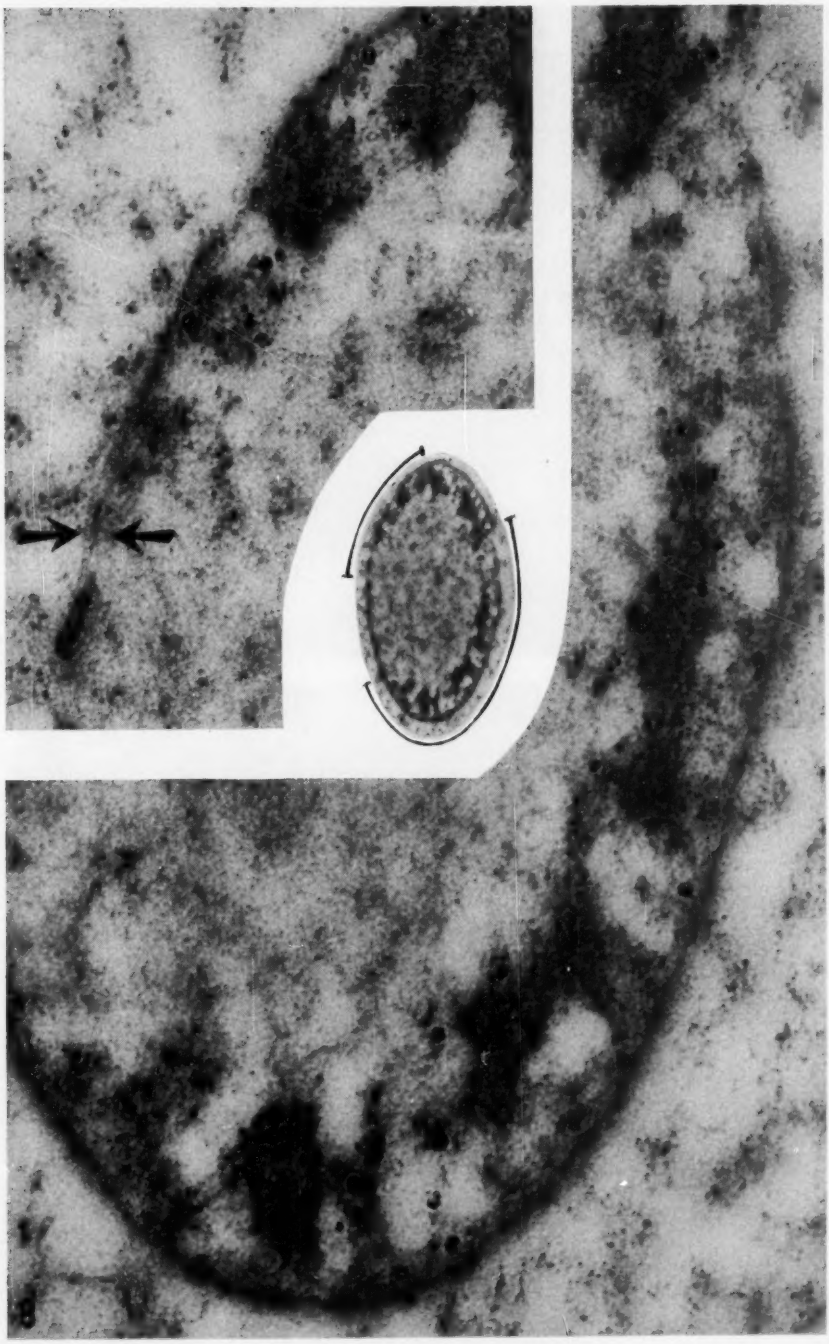
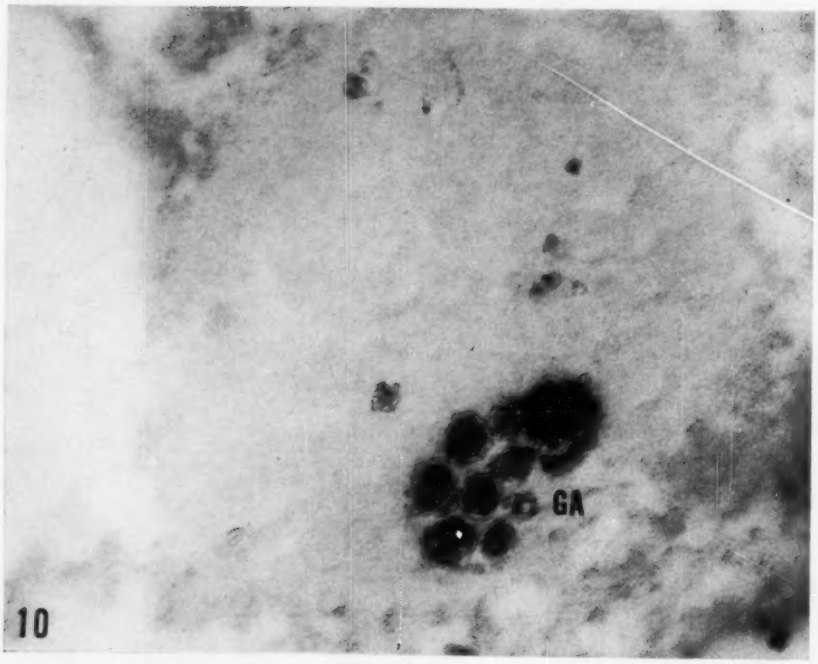


PLATE VI



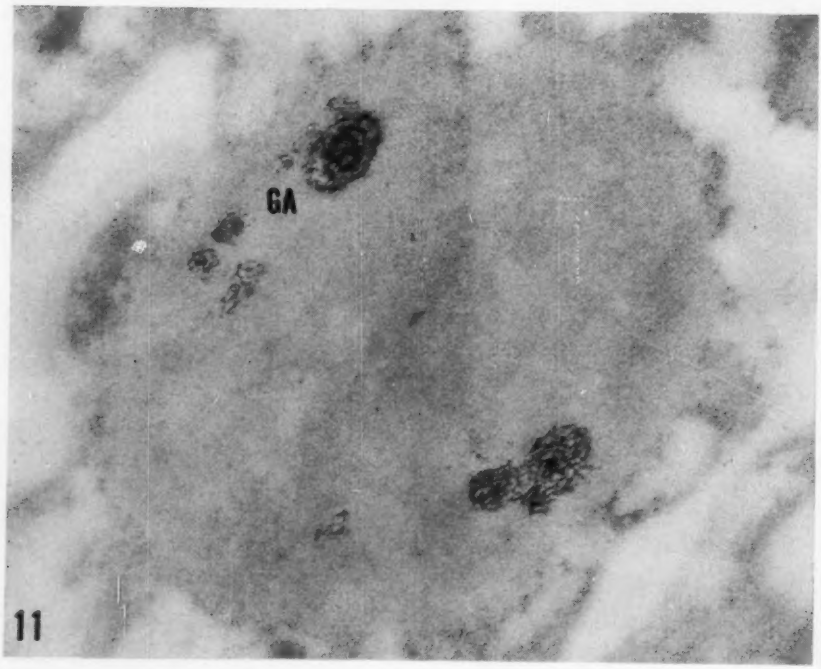
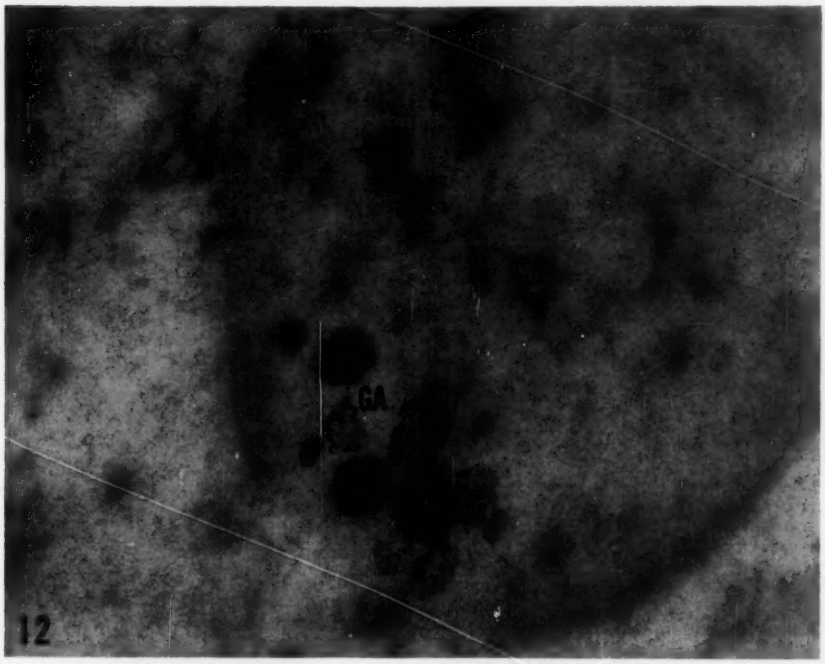
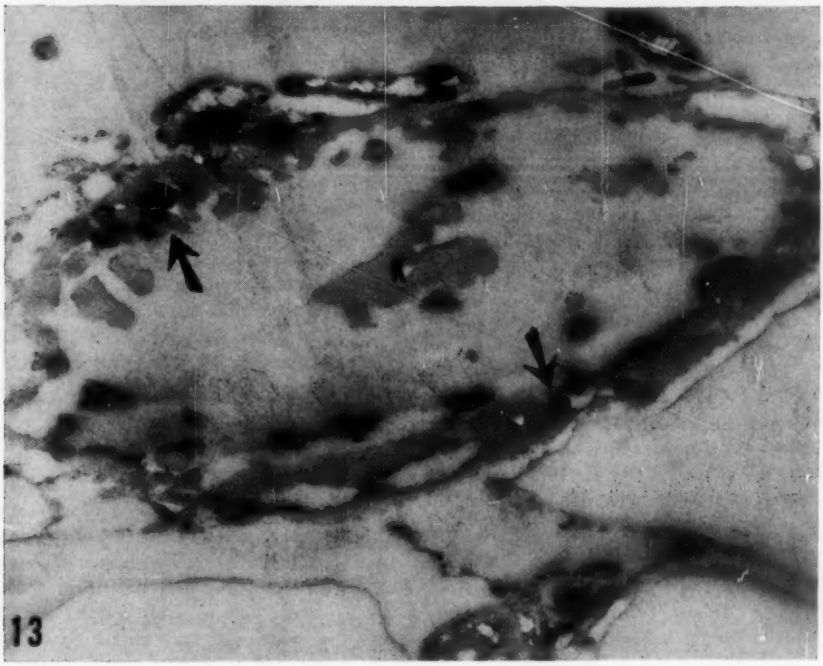


PLATE VII





A PRACTICAL METHOD FOR CULTURING COCCIDIAL OOCYSTS IN TAP WATER

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This report describes a practical technique for sporulating coccidial oocysts in plain tap water. It also describes a useful apparatus for working with large amounts of oocyst-bearing fecal material.

The use of this culture method makes special additives, such as potassium dichromate, unnecessary in sporulating, thus avoiding the possibility that this chemical may cause changes in the oocyst wall. Such changes could interfere with normal excystation of sporozoites and with normal protection of the parasites from certain environmental changes.

THE METHOD

Briefly, the method consists of removing water-soluble substances from fecal material by thorough washing and removing larger solid particles by screening. The remaining material is then centrifuged, diluted, and distributed in shallow cultures.

Step 1. As a fecal material is washed and comminuted in tap water, the solid particles are screened out with 200-mesh and 400-mesh screens. The screening process is continued until the liquid passing through the screens becomes practically clear as fresh tap water is added. The liquid and suspended material passing through the screens is collected and saved for future processing (see step 2). The material remaining on the screens is discarded.

Figure 1 illustrates the apparatus for step 1. It consists of a screenholder attached to the bottom of a cylindrical reservoir 5 inches in diameter and 12 inches in height. Above this reservoir, an electric drill motor is supported. This motor has a quarter-inch steel-bit capacity and turns a T-shaped stirrer at about 2,000 rpm. The stirrer consists of a flat blade attached at its center to a rod with the flat side at right angles to the rod (fig. 1A). The blade is a flat brass strip one-sixteenth inch thick, five-eighths inch wide, and 5 inches long. A 1-inch portion of each end of the blade is curved upward and back toward the central shaft, which reduces the over-all length of the blade to about 3 inches.

FIGURE 1. Grinding and screening apparatus for breaking up coccidial oocyst-bearing materials and for removing the larger solid particles.

Step 2. The oocyst-bearing material is freed of water-soluble substances by centrifugation. In this process, the solids flushed through the screens in step 1 are first concentrated by continuous centrifugation. This is done in a centrifuge provided with an unperforated basket

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head, 8 inches in diameter and 3.33 inches in height.* The basket head is fitted with a special

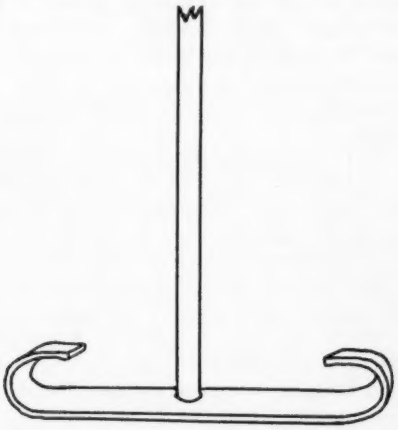

FIGURE 1A. Stirrer used in comminuting fecal material with electric drill motor, with two-thirds of rod cut off.

distributor (fig. 2), which conducts the suspended materials to the lowest and outermost areas of the basket. There, the solids including the oocysts tend to collect and remain because of centrifugal force. The distributor consists of a thick-walled metal cylinder threaded on the inside at the lower end so that it will fit on a tapered sleeve and take the place of the nut normally used to fasten the basket head to the centrifuge shaft. Communicating with the inner chamber of the cylinder are two hollow arms which radiate outward in opposite directions. Each of the arms is curved downward with the free end directed slightly backward from the direction of rotation of the centrifuge. The opening at the top of the cylinder is slightly smaller in diameter than the inner chamber of the cylinder. The passageway through the cylinder is blocked by a stopper inserted immediately below the point of attachment of the arms.

In using the basket-head centrifuge, the centrifuge is started at about 1,000 rpm. Then tap water is added until it begins to spill over the basket. A tachometer, tipped with a rubber stopper that will fit into the opening at the distributor top, is held in place over the open centrifuge. The centrifuge speed is adjusted to about 2,000 rpm. The material to be centrifuged is then run into the distributor at the rate of about 1,300 cc per minute. The liquid passing over the centrifuge basket is examined for the presence of oocysts and the speed of the centrifuge is increased if needed to prevent the loss of oocysts.

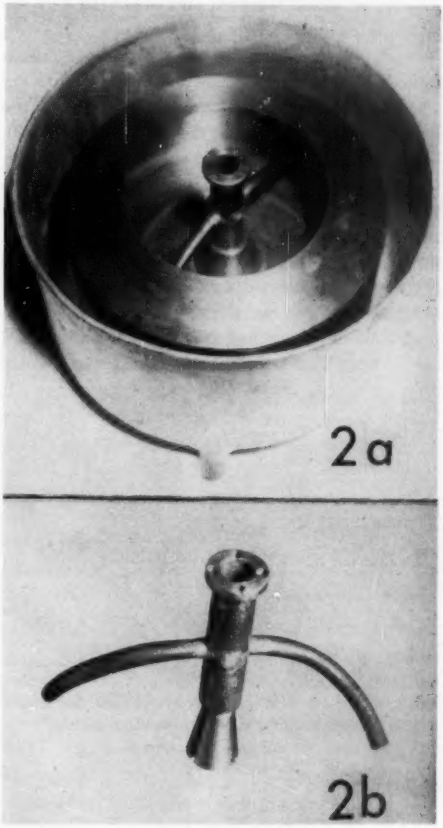

FIGURE. 2a. Centrifuge solid basket head fitted with a special distributor which improves the efficacy of the mechanism in retaining coccidial oocysts during continuous centrifugation; b, distributor attached to the tapered sleeve used in fastening the basket head to the centrifuge shaft.

The oocyst-bearing material concentrated by the continuous centrifuge is transferred to centrifuge tubes or bottles for washing, which involves a series of centrifugations and resuspensions of the solid matter in clean tap water. The washing process is continued until the super-

* Richard W. Lyons of the International Equipment Company, Boston, Mass., kindly furnished the centrifuge basket head and assisted in the first trials.

natant fluid becomes fairly clear and odorless, an important criterion for determing when the material is washed sufficiently for the cultures.

Step 3. The final step in preparing the cultures consists of reducing the washed oocyst-bearing material to a paste-like consistency in ordinary centrifuge tubes. It is then diluted for culture as described below. A small but known volume of water is added and the volume of the solids is computed from the increase in the volume of the liquid. Depending upon the abundance of oocysts in the material, additional water is added until the concentration of the oocyst-bearing solids becomes 1 to 5 cc in each 300 cc of final suspension, the greatest dilution being made where the oocyst numbers in the paste-like material is greatest. The material in the final suspension is poured into glass dishes or stainless steel pans, care being taken to make the depth of the culture material not greater than about three-eighths of an inch. These cultures, as shown in figure 3, are left uncovered and exposed to the air under laboratory conditions of temperature and light; care is taken to prevent the cultures from being exposed to direct sunlight and to unusually high temperatures. Water is added as needed to keep the material wet or moist and emphasis is placed on keeping the liquid only deep enough to prevent drying.

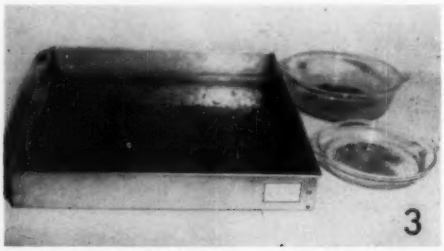

FIGURE 3. Tap-water cultures containing screened and washed oocyst-bearing materials from sheep feces. The pan is of stainles ssteel and the dishes are ordinary household pyrex baking dishes.

The time required for preparing material for sporulating oocysts in tap water is necessarily longer when no antiputrefactive agents, such as dichromate, are used. On the other hand, the rather lengthy and careful washing period needed to remove such antiputrefactive agents from oocyst-bearing material before use in experimental work is not required for materials from plain tap-water cultures.

In the plain tap water cultures, free-living ciliates and flagellates usually became fairly numerous within a few days after the cultures were set up. Occasionally rotifers and free-living amebas made their appearance. Algae developed in certain cultures of long standing, especially in those placed near fluorescent electric lights. On the whole, the presence of these other forms of living organisms do not appear to require any special attention.

SUMMARY

A technique and a certain apparatus for preparing and sporulating ovine coccidia in large batches in tap water without additives, such as potassium dichromate, are described. Oocyst-bearing material is freed of the larger solid particles through screening and of water-soluble substances by thorough washings, after which it is placed in definite amounts in shallow tap water.

ANNOUNCEMENT

SPECIAL NUMBER OF THE JOURNAL OF HELMINTHOLOGY.

A special number of "Festschrift" of the Journal of Helminthology will be published in honour of Emeritus Professor R. T. Leiper, C.M.G., D.Sc., M.D., LL.D., F.R.S., F.R.C.P., J. P., in April 1961 when he celebrates his 80th birthday. In addition to 29 original articles which have been contributed by scientists of various countries as tributes to this renowned helminthologist, there will be biographical sketches on his career and reproductions of numerous signatures to birthday greetings.

This number may be purchased from the London School of Hygiene and Tropical Medicine, Keppel Street, London W. C. I; price 30 shillings, post free. It will be issued free to subscribers to the Journal of Helminthology.

ENDOGENOUS PHASE OF THE LIFE CYCLE OF *EIMERIA AUBURNENSIS* IN CALVES*

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Eimeria auburnensis was described by Christensen and Porter (1939) from oocysts found in fecal samples from calves from Alabama, Maryland, and Montana. In one calf, discharge of *E. auburnensis* oocysts was observed to occur chiefly 24 to 27 days after inoculation with 8,000 sporulated oocysts of this species and diarrhea occurred from 9 to 13 days after inoculation. To the best of our knowledge, no other observations on the life cycle of *E. auburnensis* have been made. Over the last 15 years, one of us (D.M.H.) has occasionally observed in tissue sections coccidia suspected of belonging to this species. When an opportunity arose, experimental studies on *E. auburnensis* were begun for the purpose of obtaining information about the endogenous stages of the life cycle of this species and its effect on the host.

MATERIALS AND METHODS

Over a period of about 9 months, 21 male Holstein-Friesian calves, 1 to 6 weeks old, were inoculated with 100,000 to 750,000 sporulated *E. auburnensis* oocysts. A relatively pure inoculum (approximately 98 percent *E. auburnensis*) was first obtained from the cecum of a calf in which a cecal cannula had been placed for study of *E. bovis* infections (Hammond, Miner and Andersen, 1960). The calves were obtained locally at an age of 1 to 3 days and housed in individual 5- by 10-foot pens constructed of plywood panels. Each of the calves was fed twice daily about 0.5 gallon of raw whole milk mixed with an equal amount of water, with a vitamin A supplement. After the calves were about 2 weeks old they were given alfalfa hay and a grain mixture in addition to the milk. When the calves were about 6 weeks old, the whole milk was replaced gradually over a period of 4 to 5 days with a reconstituted milk made from skim milk powder. Fecal samples were collected at regular intervals beginning when the calves were about 2 weeks old. Daily samples were collected after inoculation of the calves with oocysts. Inoculations were done by means of nippled flasks. The samples were examined for oocysts by a modified McMaster technique (Whitlock, 1948). Three calves were killed 18 days after inoculation and 2 were killed 19 days after inoculation; mucosal scrapings were examined for coccidia and portions of intestine fixed in formalin and in Zenker's for histological study.

RESULTS

Characteristics of Oocyst Discharge and Pathogenicity

All but 2 of the 21 inoculated calves became infected. Discharge of oocysts was first detected on the 18th day after inoculation in 12 of the calves and on the 20th day in 1 calf. In 2 calves oocysts were first seen 19 days after inoculation, but no sample was taken on the 18th day. In four calves a few oocysts were found on 1 or more days from 13 to 16 days after inoculation. These discharges may have been associated with natural infections. In these 4 calves larger numbers of oocysts were discharged beginning on the 17th day in two calves, on the 18th day in one, and on the 19th day in one. In 13 calves observed during the entire period of oocyst discharge the peak number of oocysts occurred 18 days after inoculation in four, 19 days in six, 20 days in two, and 21 days in one. The period of oocyst discharge lasted 2 to 7 days.

Four calves discharged moderate to high numbers of *E. auburnensis* oocysts (approximately 14,000 to 200,000 per g of feces), with few or no oocysts of other species. Of these, one showed severe diarrhea 18 to 20 days after inoculation, two had a mild diarrhea 18 to 19 days after inoculation, and one showed no signs of coccidiosis. These findings indicate a low degree of pathogenicity for *E. auburnensis*.

Location and Development of the Sexual Stages

At necropsy of the five calves killed 18 or 19 days after inoculation, no abnormality was seen except for catarrhal enteritis of the small intestine. A marked eosinophilia was noted in the sections of small intestine. Sexual stages of *E. auburnensis* were found in mucosal scrapings of the lower two-thirds or three-fourths of the small intestine, up to 36 feet anterior to the

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ileocecal valve; the greatest concentrations usually occurred between 9 and 21 feet anterior to the ileocecal valve. In sections, gametocytes and oocysts were found to be located in cells in the lamina propria of the villi. The parasitized cells were in the peripheral portion of the lamina propria, often immediately under the epithelium. In heavy infections a single cell was frequently occupied by two parasites; in some cases these were microgametocyte and macrogametocyte.

The host cells parasitized by the gametocytes have been identified as mesenchymal-mesodermal cells rather than epithelial cells by the following pathologists: Dr. James L. Shupe, Utah State Universtiy, Dr. Cyril D. Fullmer and Dr. Bruce A. Lloyd, Holy Cross Hospital, Salt Lake City, and Dr. Lent C. Johnson, Armed Forces Institute of Pathology, Washington, D. C. Dr. Johnson stated that he suspected the mesodermal host cells may be endothelial cells, plasma cells, or small, poorly developed macrophages or large lymphocytes.

Oocysts were observed in the lamina propria in sections from calves killed 18 to 19 days after inoculation (fig. 16). Oocysts were also seen in smears of the mucosa from these calves; some oocysts were still within their host cells (fig. 17). A micropyle was seen in some young oocysts (fig. 8).

Twenty mature microgameocytes averaged 85.1 by 65.1 microns (range, 67.2 by 48.0 to 102.5 by 83.2) and each contained thousands of microgametes. In fresh mucosal smear preparations movement was seen in some of the microgametocytes. Numerous motile microgametes were observed in mucosal scrapings of four calves killed 18 or 19 days after inoculation; each microgamete had a slender, slightly curved body with two posteriorly directed flagella (figs. 12 to 15). Twenty microgametes ranged between 4 and 8 microns in length and averaged 5.5 microns, their width was about 0.5 to 0.75 micron, and the flagella were about 10 to 12 microns long.

Gametocytes of *E. auburnensis* were also observed in sections of ileum from several series of calves killed 14 to 18 days after inoculation with oocysts of *E. bovis, E. auburnensis* and other species. The gametocytes were identified by their location and by their intergradation with those seen in the sections from the 5 calves mentioned above. The earliest stages were seen in calves killed 15 days after inoculation. In one series of calves microgametocytes and macrogametocytes were distinguishable at 15 days, but in another series these could not be distinguished until 16 days. Early gametocytes (fig. 1) were in the same location as the later stages. In Zenker's-iron haematoxylin sections crescent-shaped bodies similar to those found in *E. bovis* schizonts (Hammond et al, 1946) were present at the periphery of many early gametocytes. In some macrogametocytes in which cytoplasmic granules were first becoming evident, a hemispherical or half-moon shaped body, varying in distinctness, was seen adjacent to the karyosome. Cheissin (1958) noted uniformly distributed or half-moon form concentrations of nuclear material, staining bright green with the methyl green-pyronin method, in comparable macrogametocytes of *E. intestinalis* of the rabbit.

In the series of calves in which microgametocytes and macrogametocytes could first be distinguished 16 days after inoculation the average size of the gametocytes for each day of development was determined; each average was based on 10 specimens (fixed in Zenker's, stained with iron-haematoxylin). At 15 days the gametocytes averaged 3.7 microns and at 16 days the undifferentiated gametocytes averaged 6.0 microns. At 17 days the microgametocytes averaged 22.9 microns in length, while the macrogametocytes averaged 10.3 microns. At 18 days the average length of microgametocytes was 68.9 microns and that of macrogametocytes was 17.9. Since these figures were based on only one series of calves they can be considered only as an indication of the growth trend.

The nuclei of host cells parasitized by microgametocytes became considerably enlarged and usually each contained a single prominent deeply staining spheroidal body (figs. 4, 6, 9). Often in the later stages of development, the nuclei of microgametocytes were concentrated at the surface of small spheres (figs 6, 9, 10). In microgametocytes approaching maturity, the mircogametes frequently formed whorls around residual bodies (fig. 11), as described for *E. brunetti* of the chicken by Boles and Becker (1954), and for *E. piriformis* of the rabbit by Pellerdy (1953).

Asexual stages of *E. auburnensis* have not yet been observed; investigation of these is in progress.

DISCUSSION

Eimeria auburnensis is unusual with respect to the subepithelial location of the sexual stages and the kind of cell they parasitize, as well as the large size of the microgametocytes. In *E. miyairii* of the rat there is a tendency for the parasitized cells to be pushed out of the epithelial layer toward the lamina propria, and the epithelial layer is at least temporarily broken by the passage of oocysts through it when these are eliminated from the intestinal wall (Roudabush, 1937). *E. auburnensis* differs in that its subepithelial location is apparently primary rather than secondary, and in that mesodermal cells are parasitized rather than epithelial cells. However, the discharge of oocysts into the intestinal lumen apparently involves breaking through the epithelium as in *E. miyairii*. In *E. bovis* the schizont, which attains an average size of 281 by 203 microns, develops in the center of the villi, in endothelial cells lining the lacteal (Hammond et al, 1946). Intermediate stages of *E. auburnensis* microgametocytes might easily be confused with corresponding stages of *E. bovis* schizonts, differing chiefly in their more peripheral location. Mature *E. auburnensis* microgametocytes are smaller than mature *E. bovis* schizonts and differ also in the usual presence of residual bodies. The magnitude of the microgametocytes and the large numbers of microgametes in *E. auburnensis* might be associated with the unusual location of the sexual stages, which likely makes difficult the finding of macrogametes by microgametes.

Other *Eimeria* species occur in cells of mesodermal origin, at least temporarily. Macrophages play a role in transporting the sporozoites of the chicken coccidia *E. necatrix* (See Van Doorninck and Becker, 1957) and of *E. tenella* (See Patillo, 1959; Burns and Challey, 1959) through the lamina propria of the villi to the glandular epithelium, where development of the schizonts occurs. Scholtyseck (1953) found that the sporozoites of *E. tenella* invade fibrocytes of the mucosa and submucosa and speculated that this represented a stage in evolution from an epithelial to a blood parasite, but this finding has not been confirmed by other workers (Patillo, 1959).

SUMMARY

Of 21 young calves inoculated with *Eimeria auburnensis* oocysts, 19 became infected and discharged oocysts for 2 to 7 days, beginning about 18 days after inoculation. The peak number of oocysts occurred most frequently 19 days after inoculation. In five calves killed 18 to 19 days after inoculation, catarrhal enteritis and eosinophilia were observed in the small intestine. The results of the experimental infections indicate that this species has a relatively low degree of pathogenicity.

Microgametocytes, macrogametocytes, and oocysts were found in mesodermal cells in the lamina propria of the villi in the lower small intestine. The mature microgametocytes were relatively large and each contained thousands of microgametes. Numerous motile microgametes were observed in fresh smears. Early *E. auburnensis* gametocytes were found in calves killed 15 days after inoculation with *E. bovis* and *E. auburnensis* oocysts, and intermediate stages of gametocytes were seen in calves killed 16 to 17 days after being so inoculated.

ACKNOWLEDGMENTS

The authors are indebted to Paul R. Fitzgerald and A. Earl Johnson, Animal Disease and Parasite Research Division, ARS, USDA, for their assistance in conducting the work, and to Dr. James L. Shupe, Dr. Cyril D. Fullmer, Dr. Bruce A. Lloyd and Dr. Lent C. Johnson for assistance in identifying the host cells.

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EXPLANATION OF PLATES

Photomicrographs of stages in the life cycle of *Eimeria auburnensis* from experimentally infected calves. Except where otherwise stated, all figures are of specimens in sections of small intestine fixed in Zenker's fluid and stained with iron-hematoxylin.

PLATE I

FIGURE 1. Early gametocyte in villus, with host cell nucleus at top. × 600.

FIGURE 2. Early microgametocyte, showing 3 nuclei, with host cell nucleus at top. × 600.

FIGURE 3. Early macrogametocyte, with host cell nucleus at bottom. × 600.

FIGURE 4. Early microgametocyte, showing 10 to 15 nuclei, with host cell nucleus at top. × 600.

FIGURE 5. Intermediate microgametocyte, showing 25 to 30 nuclei, with host cell nucleus at top. × 600.

FIGURE 6. Later microgametocyte, showing arrangement of nuclei at surface of spheres, with host cell nucleus at upper right. × 600.

FIGURE 7. Two later microgametocytes and four later macrogametocytes, showing granules arranged to outer wall in the three grouped specimens. × 600.

FIGURE 8. Young oocyst, with micropyle at bottom. × 600.

FIGURE 9. Nearly mature microgametocyte, with developing microgametes arranged around residual bodies, and with the enlarged host cell nucleus at the right. × 600.

PLATE II

FIGURE 10. Three microgametocytes in different stages, with the central specimen being most advanced, and four macrogametocytes in various stages. × 600.

FIGURE 11. Three microgametocytes, the one at left showing microgametes arranged in whorls around residual bodies, and the one in the center with microgametes in random arrangement. × 600.

FIGURES 12, 13, 14 15. Microgametes, each showing two flagella. Fresh specimens, phase contrast, × 1500.

FIGURE 16. Two oocysts in the lamina propria of a villus. × 600.

FIGURE 17. An oocyst inside its host cell in smear of mucosa. Fresh specimen, × 600.

PLATE I

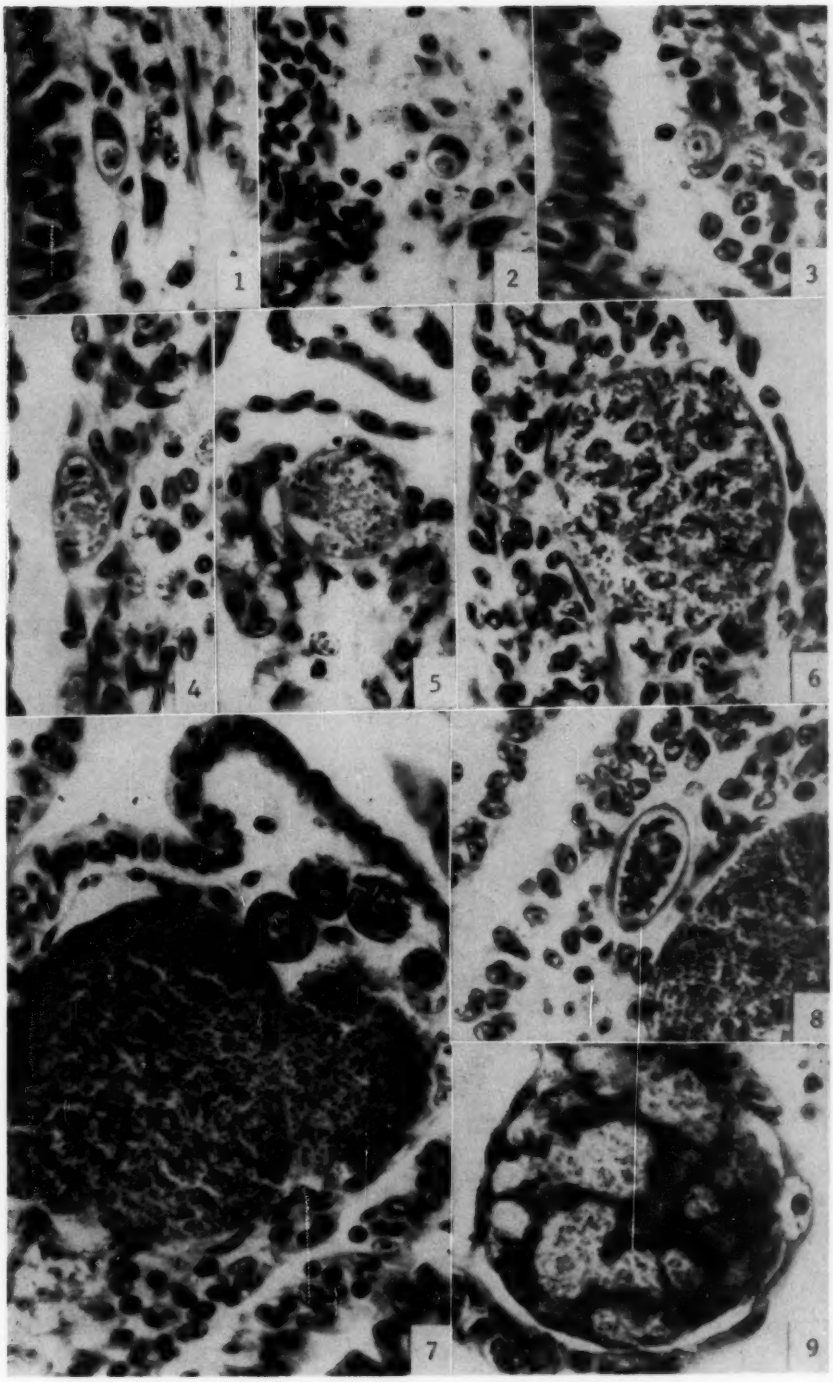
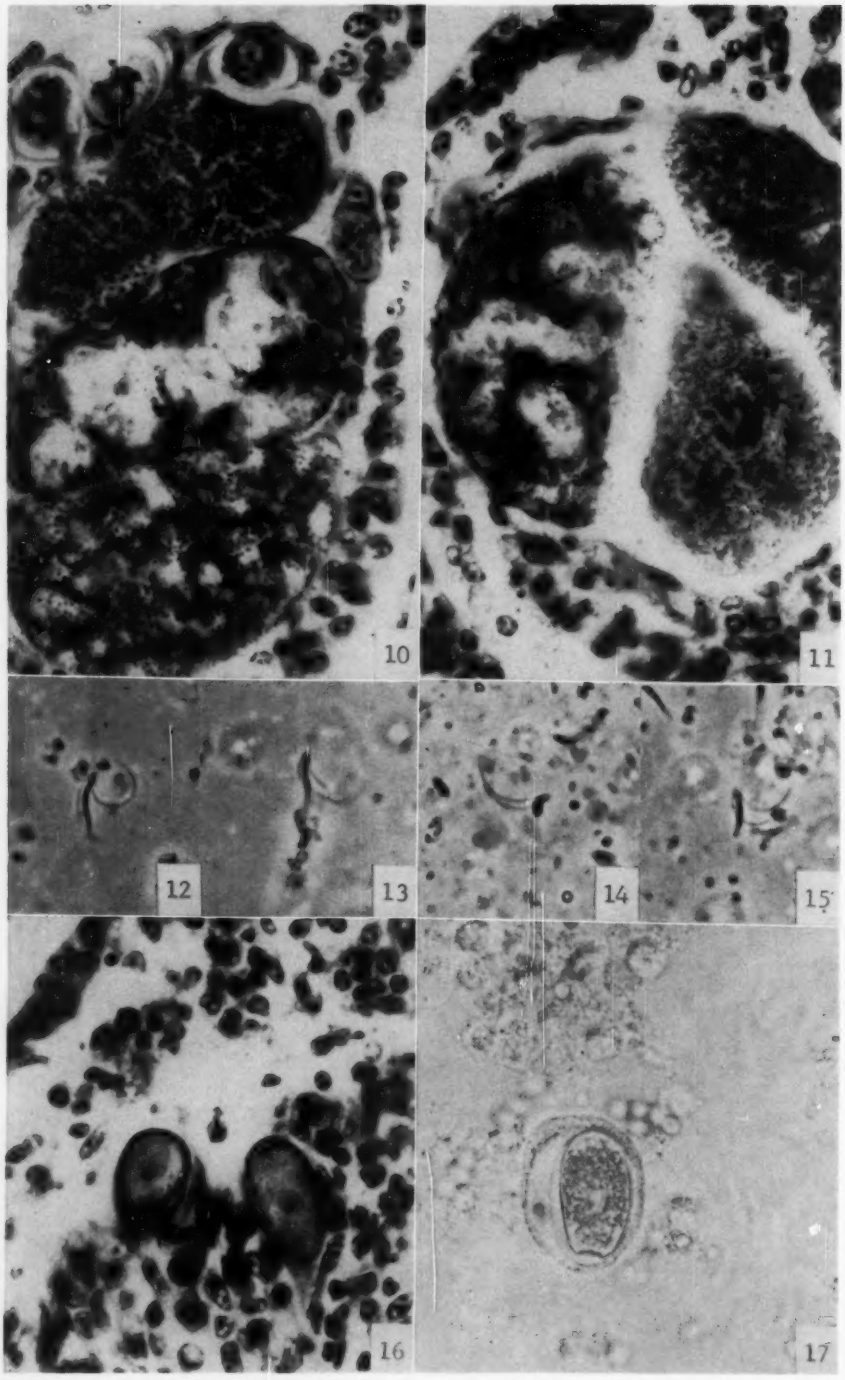


PLATE II



THE EFFECT OF THE GONADOTROPIC HORMONE CYCLE OF THE ADULT MOSQUITO ON DEVELOPMENT OF THE MALARIA PARASITE*

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Following the distension of the gut wall by the blood meal taken by an adult female mosquito, the neurosecretory cells in the brain become activated and in turn induce the corpus allatum to produce a hormone which initiates ovarian development. Interruption of this cycle by decapitation or by application of a ligature at the neck or the base of the abdomen before a critical time period will prevent ovarian development (Clements, 1956; Detanova, 1945; Gillett, 1956; Larsen and Bodenstein, 1959). Since the malarial oocysts, like the ovaries, grow within the hemocoele of the mosquito and therefore draw upon nutritive and growth-promoting substances in the hemocoele, the possibility presents itself that the host hormones may also affect the parasite. The purpose of this study was to determine whether interruption of the hormone cycle involved in ovarian development would also affect the growth of the malarial oocysts.

MATERIALS AND METHODS

The experimental organisms were *Aedes (S.) aegypti* L. and *Plasmodium gallinaceum* Brumpt. Female mosquitoes were decapitated after varying periods of time following engorgement upon an infected chicken. The time between engorgement and decapitation varied from 1 minute to 6 hours. Of several methods attempted, the most successful for survival of the decapitated insects involved insertion of a finely drawn out capillary pipette into the thorax through the severed neck. The tube, with the mosquito at the end, was then taped to the inside rim of a cardboard cylinder which was placed on a dish of sand moistened with a solution of mold-inhibiting Tegosept. Prior to insertion, the capillary tube was filled with a 50–50 mixture of 10 percent sucrose and 10 percent rabbit serum-saline containing streptomycin and penicillin. (The RSS medium was a standard tick- or mosquito-grinding fluid for virus isolations containing 200 units of penicillin and 200 micrograms of streptomycin per ml.) It is uncertain whether the sugar contributed to the mosquitoes' survival or whether the penicillin and streptomycin prevented bacterial growth. Since, as will be shown below, oocysts developed in decapitated mosquitoes, the antibiotics did not appear to affect the malarial parasites. Control insects were placed in holding cages, and were given no additional food.

Dissections were made in the third, fourth, and fifth day following the infectival meal, at which time the oocysts were counted and measured. Care was taken to prevent drying of the preparation so that the oocyst size would not be affected by pressure of the coverslip.

RESULTS

The total infection rates of the two groups of mosquitoes are presented in table I. About one-third more of the normal mosquitoes were infected. Although there was no difference in infection rates between the two groups in some experiments, it appears that decapitation did prevent infection of a considerable proportion of the insects.

TABLE I. *Total mosquito infection rates.*

Exp. No.	Decapitated mosquitoes			Normal mosquitoes		
	No. dissected	No. positive	Pct. positive	No. dissected	No. positive	Pct. positive
4	4	3	75	4	4	100
5	8	6	75	11	9	81.8
17	4	2	50	9	9	100
25	2	2	100	2	2	100
27	2	2	100	5	1	20
28	4	1	25	2	2	100
29	17	6	35.3	34	32	94.1
30	20	12	60.0	10	9	90
33	7	3	42.9	7	6	85.7
35	14	11	78.6	5	4	80
Totals	82	48	58.5	89	78	87.6

A summary of the numbers of oocysts per infected mosquito is given in table II. The large standard deviation illustrates the great variability in numbers of oocysts among mosquitoes of a given batch when fed at the same time on the same host. In general, there were fewer oocysts in the decapitated than in the normal mosquitoes. The difference was calculated to be significant in only 2 of the 6 experiments in which at least 3 mosquitoes of each group were dissected.

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TABLE II. *Numbers of oocysts in infected individuals.*

Exp. No.	Decapitated mosquitoes				Normal mosquitoes				$\bar{x}$d**	SEd	p
	No. mosq.	Oocysts			No. mosq.*	Oocysts					
		Total	$\bar{x}$	s		Total	$\bar{x}$	s			
4	3	20	6.7	5.1	4	122	30.5	29.0	+ 23.8	17.3	> 0.1
5	6	98	16.3	12.7	9	475	52.8	33.6	+ 36.5	15.7	0.05
17	2	35	17.5	—	9	154	17.1	15.6	− 0.4		—
25	2	9	4.5	—	2	60	30.0	—	+ 25.5		—
27	2	38	19.0	—	5	90	18.0	—	− 1.0		—
28	1	5	5.0	—	2	410	205.0	—	+ 200.0		—
29	6	250	41.7	53.4	8	395	49.4	45.3	+ 7.7	26.4	> 0.5
30	12	262	21.8	26.8	9	254	28.2	25.6	+ 6.4	11.6	> 0.5
33	3	106	35.3	43.9	6	146	24.3	24.6	− 11.0		—
35	11	59	5.4	3.6	4	176	44.0	38.0	38.6	10.8	< .01

* Table 1 shows greater numbers in this column because of inclusion of infected specimens in which oocysts were not counted.

** $\bar{x}$d, difference between means; +, normal greater than decapitated; −, normal less than decapitated; p, calculated from t-test.

The largest number of oocysts counted in a decapitated mosquito was 150; in normal mosquitoes it was 242. There was no correlation between the numbers of oocysts and the length of time between engorgement and decapitation.

A comparison of growth of the oocysts in the two groups of mosquitoes is presented in table III. It is seen that in the decapitated specimens in which blocking of the hormone cycle prevented ovarian development, there was continued growth of the oocysts during the 3- to 5-day observation period. It should be noted that in the 48 decapitated insects which were infected, in only 8 had the ovaries progressed beyond the diapause state. Of these, 4 were in Christophers' stage III, 2 in stage IV, and 2 in stage V. One of the insects with stage III ovaries had been decapitated 30 minutes after engorgement; for 2 others this interval was about 1.5 hours; for the remaining it ranged from 2.5 to 6 hours. There was no enhancement of oocyst growth in these eight insects. Thus it is evident that the hormonal requirements essential for initiation and continuation of ovarian development are not utilized by the malaria parasite. There was a significant delay in the rate of growth of oocysts in the decapitated insects, which was especially noticeable by the fifth day. Other than their smaller size, these oocysts exhibited no visual evidence of abnormality.

TABLE III. *Comparison of oocyst growth in microns: decapitated vs. normal mosquitoes.*

Day	Decapitated				Normal				$\bar{x}$ diff.	p
	No. oocysts	Mean diam. ($\bar{x}$)	s	SE$\bar{x}$	No. oocysts	Mean diam. ($\bar{x}$)	s	SE$\bar{x}$		
3	356	8.6	2.5	0.1	910	11.1	2.7	0.1	2.5	< .01
4	361	9.7	2.1	0.1	592	13.9	2.5	0.1	4.2	< .01
5	174	14.7	2.3	0.2	322	24.2	6.1	0.3	9.5	< .01

DISCUSSION

Many factors appear to be involved in the host-parasite specificity exhibited by mosquitoes and malaria plasmodia. Parasites must borrow from the host, and the degree of specificity may depend upon the needs of the parasite for the host's enzymatic or other physiological processes. It is not unreasonable to suspect that hormones produced by the host may contribute to the welfare of the parasite.

Interruption of the gonadotropic hormone cycle in *A. aegypti* females did not prevent the growth of *P. gallinaceum* oocysts; however, decapitation did cause reduction in the numbers of infected mosquitoes, in the intensity of infection, and the rate of growth.

Recently, Howard (1959) has questioned the role of ookinete motility as a factor in the invasion of the gut epithelium of the mosquito by the malaria parasite. According to this author, the parasite is passive, while the changes in the shape of the epithelial cells as the gut contracts about the blood meal results in entrapping certain zygotes. Decapitation must cause many changes in normal physiological processes. Although we have no direct evidence that a marked reduction in contractility of the gut wall follows decapitation, this may at least be considered as

a possible explanation for the reduction in the numbers of parasites successfully invading the gut epithelium. If this invasion is due to the movements of the ookinete, one might expect no difference in the infection rates or in the intensity of infection of the decapitated and normal mosquitoes. On the other hand, blood digestion does proceed in the decapitated females which survive 3 to 5 days. In many specimens a residue of the meal may remain in the gut; in others the gut becomes completely empty as in normal mosquitoes. Thus a sufficient degree of normal digestive processes, including changes in the epithelium, continues to take place in decapitated mosquitoes to permit a somewhat reduced number of zygotes to be taken up by the epithelium. It must be conceded that some other obscure biochemical factor may be responsible for both fewer invading zygotes and retarded oocyst growth.

The delay in growth of the oocysts in decapitated mosquitoes is thought to be due to a more rapid depletion in nutritive or other materials required by the parasites. In view of the activity displayed by some of these mosquitoes, even after 5 days of decapitation, it would be of interest to know just what has been depleted. The control mosquitoes were given no food or water, so that their reserves could not be replenished. Hence, it is not impossible that in addition to the supply of amino acids, sugars, and other food materials, the normal mosquito is contributing accessory factors which are necessary for normal growth of oocysts.

SUMMARY AND CONCLUSIONS

Interruption of the gonadotropic hormone cycle in *Aedes aegypti* by decapitation following engorgement on chickens infected with *Plasmodium gallanaceum* did not prevent the growth of the oocysts. A lower rate and intensity of infection in the decapitated mosquitoes may be due to reduction in contractility of the gut wall during the digestion of the blood meal. The slower rate of growth of oocysts in the decapitated mosquitoes is thought to be due to more rapid depletion of food materials, although the possibility that accessory growth promoting substances are furnished by the normal mosquito cannot be excluded entirely.

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RESEARCH NOTE

A NEW RECORD OF CERATOPOGONIDAE (DIPTERA) FEEDING ON FROGS.

One of the writers (Pechuman) has found an excellent way to collect a representative sample of Tabanidae by locating a small area of open water on a narrow, slowly moving stream otherwise largely concealed by brush. Blind sweeping of a net over such spots has resulted in as many as 16 species of Tabanidae being collected in an hour. A favorite spot for such collecting is a log bridge over the 6-foot-wide outlet of Day's Lake a few miles south of Seguin Falls, Parry Sound District, Ontario. Except at this spot the stream is largely concealed by a dense growth of *Myrica gale*. Discarded tabanids dropped in the stream are eagerly seized by frogs (probably *Rana pipiens*) resting on partly submerged branches projecting from the bank. These frogs become remarkably tame and are not disturbed by a net swinging an inch or less from their exposed heads.

Pechuman noted in July 1960 that numbers of small midges were in constant motion around the heads of these frogs and occasionally one or more settled on a frog's head. The frog exhibited signs of irritation by rapidly blinking its eyes and sometimes by kicking at the top of its head with a hind leg. We assumed that the flies were biting the frogs. Occasionally a frog submerged when attacked and the cloud of midges would continue to gyrate over the spot for some time.

On August 27, 1960, an attempt was made to collect some of the midges. The frogs permitted the net to be swung a fraction of an inch over their heads, but although six or eight midges could be collected at a time, they would escape through the nylon mesh before they could be secured. Finally, however, three specimens were collected. These were identified by Wirth as *Forcipomyia* (*Lasiohelea*) *fairfaxensis* Wirth, family Ceratopogonidae. This species was described from Virginia and is known from Ontario to Michigan, Massachusetts, and Virginia. It is common in swampy woods where it is frequently collected by sweeping vegetation, but heretofore its feeding habits were unknown. The known larvae of other species of the subgenus have been found next to wet soil on wet, rotting pieces of wood.

Desportes and Harant (1940, Ann. Parasit. **17**: 369–374) described the feeding habits of a related species, *Forcipomyia* (*Lasiohelea*) *velox* (Winnertz), feeding on frogs at the edge of a pond in France. In 1941 (id. **18**: 46–67) and 1942 (id. **19**: 53–68) Desportes in France worked out the life cycle of the filarial worm *Icosiella neglecta* Diesing in *F. velox*. Larval development was noted in 60.5 percent of the midges experimentally fed on infected frogs, and 5.3 percent of the midges caught on frogs in nature contained infectious filarial larvae. By a series of tests Desportes found that from 22 to 26 days were necessary for larval development to the infectious stage in *Forcipomyia*. Specimens of *F. velox* were experimentally induced to feed on all batrachians presented to them, but would not feed on reptiles or eels, or on such warm-blooded hosts as rabbits, guinea pigs, rats, chickens or pigeons. These midges were caged successfully for transmission tests for periods as long as 25 to 30 days after blood-feeding on frogs, by keeping them outdoors in tubes closed with bolting cloth on cork floats sheltered from sun and rain, on the surface of a pond where natural conditions of temperature and humidity prevailed. Other bloodsucking Diptera were also, but only rarely, caught on the frogs: *Culex apicalis* Adams and *C. hortensis* Ficalbi, Culicidae, and *Atrichops crassipes* (Meigen), Rhagionidae, in which filarial development was negative, and *Sycorax silacea* Curtis, Psychodidae, in which filariae were observed in various stages of development.

We are indebted to Roy C. Anderson, Department of Parasitology of the Ontario Research Foundation in Toronto, for the following information on North American records of *Icosiella*. Walton (1935, J. Parasit. **21**: 27–50) reported *I. quadrituberculata* (Leidy) in *Amphiuma tridactylum*, *Rana catesbiana* and *R. pipiens*. In 1947 (J. Parasit. **33** (supp.): 26–27) Walton reported this species again from *R. catesbiana*. The collection of the U. S. National Museum at Beltsville, Maryland, has specimens of *I. quadrituberculata* from *Rana pipiens* (No. 4564 E Mo.) and the "River swamp frog" (No. M599C, N.I.H.).

The attention of American parasitologists is invited to the possibility of *Forcipomyia fairfaxensis* acting as the vector of the American frog filaria. In view of the common and widespread occurrence of this midge in eastern North America, together with Desportes' success with his well-designed technique for transmission studies with these small insects, the possibilities for successful experimental work are very inviting. Although other ceratopogonid midges of the genus *Culicoides* have been incriminated as vectors of the filarial worms *Mansonella ozzardi* (Manson), *Acanthocheilonema perstans* (Manson), *Dipetalonema streptocerca* (Macfie and Corson), *Onchocerca cervicalis* Railliett and Henry, and *O. gibsoni* (Cleland and Johnson), the difficulties in handling these midges in the laboratory have seriously handicapped transmission studies. The experience gained in transmission work with *Forcipomyia* and *Icosiella* may provide valuable leads in developing more effective techniques for handling other blood-sucking midges such as *Culicoides* and *Leptoconops* in vector studies. Moreover, species of *Forcipomyia* (*Lasiohelea*) are serious bloodsucking pests of man and other warm-blooded animals in the tropics. Their role as possible filarial vectors need investigation.—L. L. PECHUMAN, *Lockport, N. Y.*, AND W. W. WIRTH, *Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.*

EFFECT OF A DIET DEFICIENT IN FACTOR 3, VITAMIN E AND CYSTINE ON THE COURSE OF *PLASMODIUM BERGHEI* INFECTIONS IN MICE

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Many experiments have been performed testing the effect of various dietary deficiences in the host on the course of malarial infections. Deficiencies of one kind or another were found (a) to increase or (b) decrease the severity of the infection or (c) were without marked influence on the course of the infection.

(a) Marked increases in parasitemia due to *Plasmodium lophurae* were obtained in biotin- (Trager, 1943, 1945, 1947; Seeler, Ott and Gundel, 1944; Seeler and Ott, 1946), "folic acid"-, and probably also vitamin B^{12}- (Seeler and Ott, 1945, 1946), and nicotinic acid-deficient chicks (Ross, Hegsted and Stare, 1946); but a nicotinic acid deficiency in ducks infected with *P. lophurae* produced no effect on the parisitemia (Ross, Hegsted and Stare, 1946).

(b) A deficiency in riboflavin depressed parasitemia with *P. lophurae* in chicks (Seeler and Ott, 1946). A pantothenic acid deficiency markedly depressed *P. gallinaceum* (Brackett, Waletsky and Baker, 1946) but not *P. lophurae* (Trager, 1945) in chicks. *P. gallinaceum* could also be depressed by chemical analogues of pantothenic acid (Brackett, Waletsky and Baker, 1946; Deschiens and Pick, 1949). *p*-aminobenzoic acid deficiency has been tested indirectly, its absence from the diet being one of the probable explanations for the depressing effect of milk diets in malaria of mammals (Hawking, 1953, 1954) and, of course, the relationship between the antimalarial activity of sulfonamides and *p*-aminobenzoic acid is well documented. Other dietary deficiencies reported to depress malaria are vitamin A (*P. lophurae* in chicks, Ross, Hegsted and Stare, 1946, and *P. berghei* in rats, Fabiani and Grellet, 1952), and ascorbic acid (*P. knowlesi* in monkeys, McKee and Geiman, 1946).

(c) Even a severe thiamin deficiency did not alter the course of *P. lophurae* infections in chicks (Seeler and Ott, 1946) and a choline deficiency produced equivocal results (Ross, Hegsted and Stare, 1946).

The effect of vitamin E on malarial parasitemia has been tested indirectly in that it will reverse the depressing effect of a cod-liver oil containing diet on *P. berghei* in mice, as will other antioxidants such as ascorbic acid and tetraethyl thiuram disulfide (Godfrey, 1957). There have been no reported tests, which have come to our attention, on the effects of deficiences in pyridoxine, vitamin B_{12}, D or K on malaria. The only study with amino acids appears to be that of McKee, Geiman and Cobbey, 1947, and McKee and Geiman, 1948, who found that in starving monkeys *P. knowlesi* infections were depressed and this depression could be reversed by feeding methionine. Methionine did not reverse the supperssive effect of starvation on *P. berghei* in rats (Dutta and Chaudhuri, 1953). There have been no reported tests of the effect of a deficiency in Factor 3 on any malarial infection.

A Schwarz *Torula* yeast diet produces liver necrosis in rate (Schwartz, 1951, 1951a), multiple necrosis of various organs in mice (DeWitt and Schwarz, 1958), and exudative diathesis in chickens (Scott, Hill, Norris, Dobson, and Nelson, 1955). The diet used to produce these lesions is based on dried American *Torula* yeast and is deficient in cystine and vitamin E as well as Factor 3. Factor 3 is obtained, among other sources, from dried defatted, pork kidney powder and contains organically bound selenium (Schwarz and Folz, 1957). The symptoms are relieved by feeding the animals cystine and vitamin E, or inorganic selenium as well as Factor 3 (Schwarz and Folz, 1957; Schwarz, Bieri, Briggs, and Scott, 1957).

DeWitt (1957, 1957a) observed that in mice fed a Factor 3-deficient diet *Schistosoma mansoni* did not attain normal adult size and very few reached sexual maturity. Supplementing the deficient diet with vitamin E and cystine together or Factor 3 alone gave results comparable with mice maintained on a complete diet.

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The present paper reports the results of experiments designed to test the effect of a diet deficient in Factor 3, vitamin E, and cystine on the course of *Plasmodium berghei* in mice.

MATERIALS AND METHODS

Mice used in these experiments were F_1 progeny from homozygous strains of BALB/cAnN females and DBA/2JN males. Starting at term, the mother's diet was restricted in vitamin E content. The young mice were weaned at 4 weeks, and placed in individual wire-mesh cages. Water and food were given ad libitum. The diets were prepared in dry form and offered to the animals from glass cups.

Fifty animals, 25 of each sex, were placed on the normal diet and a like number, also divided equally as to sex, were offered the test diet. After 4 weeks on these diets, the first group of animals (12 males and 12 females on the test diet and 12 males and 12 females on the normal (control) diet) was inoculated i.v. with 1 M *Plasmodium berghei* (Kasapa strain) from a white Swiss donor. Ten days later, or after 38 days on the diets, the second group (9 males and 13 females on the test diet and 13 males and 12 females on the normal (control) diet) was inoculated in the same manner with the same parasite as the first group.

The composition of the test diet was described by Schwarz, 1951 and 1951a. The main ingredients in percent are: *Torula* yeast 30, sucrose 59, vitamin E-free lard 5, salts 5, and a vitamin mixture (in lactose) 1, vitamin A acetate (1 mg percent and vitamin D_2 (1 mg percent). The composition of the normal, or control, diet was casein "vitamin-free" 30, sucrose 44.7 hydrogented vegetable oil, (Crisco) 17, salts 4, Wesson oil plus vitamins A and D_2 2, Wesson oil plus vitamin E 1, and a mixture containing all the known B vitamins plus K 1.

Smears of the peripheral blood were made from each mouse daily, beginning on the day following infection, and the preparations stained with Giemsa stain. Each day's parasitemia was expressed as two values, (1) percent of total RBC infected and (2) percent of mature RBC infected. Day of death was tabulated in all instances.

RESULTS

A summary of the data on parasitemia is shown in the figure and survival data are given in the table. There appeared to be no significant difference between the sexes in any diet group so the data on parasitemia of both sexes were pooled. The only difference in parasitemia between mice on the complete diet and those on the test diet appeared in the first week. Thereafter, the differences, if any, were trivial. The differences which occurred in the first week can be entirely accounted for in terms of the infection in mature erythrocytes. In mice fed the normal diet the infection in mature erythrocytes reached a maximum of 10 to 15 percent at the end of the first week and then declined. In mice fed the test diet the infection of mature erythrocytes did not exceed 5 percent on any day. The maximum infection of mature erythrocytes in individual mice on the normal diet ranged from 5.5 to 20 percent; in mice on the test diet the range was 1.5 to 11.7 percent. Combining the data for both series one finds that the highest infection of mature erythrocytes, regardless of the day on which it was observed, averaged 5.1 for mice on the test diet and 12.6 for mice on the normal liet.

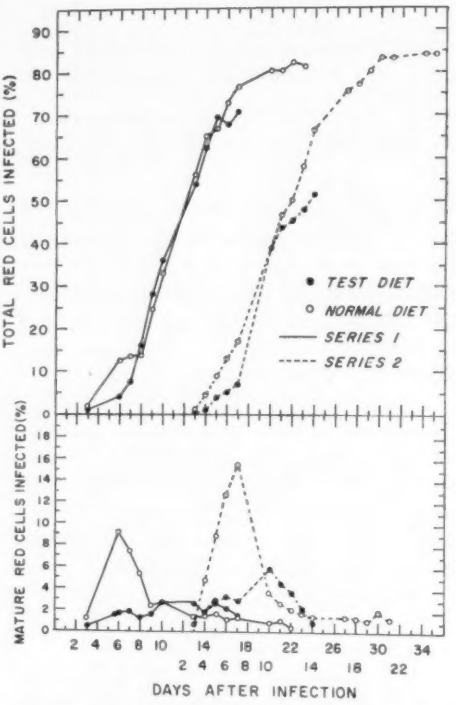

FIGURE 1. Course of *Plasmodium berghei* infection in BALB/cAnN × DBA/2JN F_1 mice fed a complete or a Factor 3-deficient diet. Points are the arithmetic means of the parasitemia of all mice by day and by diet.

Even though the differences in parasitemia appeared to favor the mice on the test diet, the mice on the normal diet survived longer (table I). In neither the control nor the experimental diet group was there any large difference in survival between males and females.

There were no major differences in the survival between series 1 and series 2 even though those in series 2 were on the deficient diet 10 days longer. Combining the data for both sexes and both series, the mice on the deficient diet lived 15.5 days, those on the complete ration 22.9 days. However, all mice on the deficient diet survived well into the second week of the infection.

TABLE I. *Survival time in mice fed a diet deficient in Factor 3 or a complete diet and infected with the Kasapa strain of* Plasmodium berghei.

Series	Factor 3 deficient diet		Complete diet	
	Males	Females	Males	Females
1*	15.8 (13–20)	15.9 (12–21)	21.8 (19–23)	22.0 (20–26)
2**	15.1 (11–16)	15.5 (10–17)	23.5 (23–25)	24.4 (20–29)

* On diet 28 days before infection with *P. berghei.*
** On diet 38 days before infection with *P. berghei.*
Figures in parenthesis are ranges.

DISCUSSION

The test diet had two effects in mice infected with *P. berghei:* (1) It suppressed the infection in mature erythrocytes and (2) it reduced survival time by 7 days. After the first week the parasitemia, as observed in blood smears, is mainly a reflection of the infection of immature erythrocytes which become preponderant in the erythrocyte population (Greenberg and Coatney, 1954). There were no significant differences between the two groups of mice after the first week. It may be concluded that the deficiency produced by the test diet did not materially influence the infection of immature erythrocytes.

In most strains of mice there is a major wave of deaths at the end of the first week. This wave of death coincides with a crisis in the infection of mature erythrocytes and an acute hemolytic crisis. Death has been attributed to shock resulting from hemolysis (Highman, Greenberg and Coatney, 1954). Mice which survive this initial wave of death die subsequently of anoxia. Some hybrid mice have been found refractory to causes of death in the first wave. This seems to be true of the hybrid used in the current series and the test diet did not increase the susceptibility of the mice to death during the first wave. The earliest death in mice on the test diet occurred on day 10 and half of the mice survived the 15th day. The mice on the test diet were, however, more susceptible to secondary causes of death. According to DeWitt and Schwarz (1958), BALB/cAnN × DBA/2JN F_1 hybrids fed the Factor 3-deficient diet survived from 38 to 96 days, averaging about 70 days. Deaths in both of the series of the present investigation could have been caused by dietary deficiency alone. However, in the first series they died about 27 days earlier than the average expected deaths from diet alone and in the second series they died 17 days earlier than would have been expected from diet alone. The fact that in both series the average survival was similar would imply that the major cause of death was malaria. The fact that deficient mice with malaria died a week earlier than would have been expected from malaria alone would imply that the dietary deficiency placed an additional stress on the malarious mice. That deaths occurred chiefly during the second and third week of the infection would imply that the dietary deficiency did not materially aggravate the causes of death associated with the first wave of death in these mice.

It remains to be seen whether the effects observed with Factor 3-deficient mice are reversible by cystine, vitamin E or selenium. The relationship between Factor 3-deficiency and suppression of the infection in mature erythrocytes is obscure as is the additive effects of the toxicity of malaria and Factor 3-deficiency.

SUMMARY

BALB/cAnN × DBA F_1 male and female mice were fed a diet known to produce multiple necrotic degeneration of various organs because of a deficiency in Factor 3, cystine and vitamin E. Control mice of the same genetic background were fed an adequate diet. All mice were inoculated with *Plasmodium berghei.*

The experimental mice had less than half as many infected mature erythrocytes (5.1 percent) at the end of the first week as the controls (12.6 percent). There were no obvious differences between the two groups in the course of the infection in mature erythrocytes. Control mice survived, on the average, 23 days after inoculation with malaria; deficient mice, 15 days.

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THE RELATION OF RABBIT TICK POPULATIONS TO SPACING IN HOST POPULATIONS

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Results of comparative studies of host and ectoparasite populations indicate indirectly that spacing between individual hosts is an important factor governing the density of the ectoparasite populations. Rat-flea populations have been reported to be roughly proportional to the density of their host populations (Eskey, 1930). Declining populations of snowshoe hares, *Lepus americanus*, have been shown to induce declines in populations of the bird and rabbit tick, *Haemaphysalis leporispalustris*, (Green, Evans, and Larson, 1943). The decline in hare populations probably reduces overlapping of activity, thus leaving increasing proportions of ticks without hosts.

Although spacing is difficult to measure in motile animals, such study has been facilitated by a statistical method developed by Hayne (1949) for determining centers of activity, and by Dice (1952) and others for determining distance to nearest neighbors. These permit measurement of spatial relationships between individuals as well as between aggregations. Presumably such spacing affects arthropod-borne diseases as well as the parasites.

The present study utilizes data previously gathered (Stannard and Pietsch, 1958) on the dog tick, *Dermacentor variabilis*, and the bird and rabbit tick for the purpose of determining the relation of these ectoparasites to spacing in cottontail rabbit populations in Lee County, northern Illinois. The field work was done under direction of Dr. Thomas G. Scott who encouraged the collection of comparative data on host and ectoparasite populations.

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METHODS AND EXPLANATION

Methods of studying the populations of ticks and rabbits are described by Mohr and Lord (1960). Rabbits were caught alive, marked, searched for ectoparasites, and released for future observation.

The infestation by ticks on individual rabbits are here given as deviations from the average number of ticks per rabbit for the pertinent month as reported by Stannard and Pietsch for Lee County (1958, table II). Their monthly averages are called base data. No ticks were found in January, February, and December.

Area I, described by Mohr and Lord (1960), is treated as two areas: A and B (fig. 1). Area A was uniformly trappable because it was not disturbed by plowing and cultivation. Other portions were under cultivation during much of the crop-growing season or otherwise inaccessible for uniform arrangement of traps if under small-grain or hay crops. Because it is difficult in such situations to measure objectively how much of an area has in fact been studied, the center of each area over which traps were placed was calculated, then the area was calculated within which 75 percent of the traps lay. Seventy-five percent of the traps on area A were on 7 acres, and on areas B and II were on 213 acres. The number of trap-nights per acre was 419 on area A and 272 on each of areas B and II. An appraisal of the habitat and rabbit population surrounding the study areas indicates that spacing of rabbits was more similar to that on areas B and II than to that on A.

Because rabbits were being born, dying, and apparently drifting or migrating across the study areas, and because some evaluation of observed use is desirable, a number was assigned to each rabbit representing the number of days from the first day caught through the last day caught. Those caught only once got a value of one. The number of rabbit-days of observation was 25,317 on area A and 9,024 on areas B and II.

Centers of activity were calculated according to the procedure recommended by Hayne (1949) for each rabbit that was caught in more than one trap. In cases in which individual rabbits were caught over a period longer than 365 days, a center of activity was calculated for each 365-day period or fraction of this period.

Areas also were calculated within which 50 percent of the catches of each rabbit were made. These are shown by the series of circles in figure 1. Although trapping was done during 3 years (February 1952 through November 1954), the data

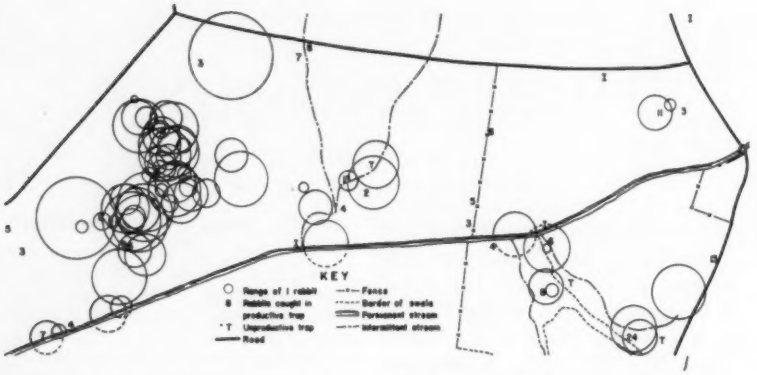

FIGURE 1. Area I showing sub-area A (cluster of circles to the left) in which rabbits were most concentrated, and sub-area B (circles outside cluster) where the rabbits were more widely dispersed. The area is 0.59 miles long.

are treated as a unit (fig. 1) as though from a composite population. This treatment is believed to be justified because the population of the bird and rabbit tick in one season partly determines the size of the population in the next (Green et al, 1943), and because more data thus become available for a given area or a given month. The total number of rabbits caught only once or in only one trap is shown at each trap station. Although by coincidence, the number of rabbits caught (497) during the 3-year period was not much different from that (355 to 481) estimated to be present per year.

Distance between observed centers of activity was measured by two different methods. The first consisted of finding the distance from center of activity of each individual to that of the next nearest neighbor, then from the latter to the next nearest and so on until the distance between every center and the next nearest was determined as suggested by the work of Clark and Evans (1954).

The second method was as follows: Each circle of activity (fig. 1) was divided into four quadrants following Dice (1952) who, however, divided the circle into six sections. Then the distance was measured from each center to the next nearest center in each quandrant except when, as in the case of rabbits at the margin of the study area (fig. 1) there was no observed neighbor. In such case, the distance not yet measured to the nearest one in any other quadrant was substituted. There are then four measurements for each rabbit that was caught more often than once. These distances were averaged.

Eighty rabbits for which centers of activity could be calculated were examined for ectoparasites.

There was an unmeasured amount of equalization of the tick population by birds which are hosts of larval and nymphal stages which drop from the birds and reinfest rabbits. General observations indicate that the birds were concentrated in much the same proportion and in the same areas as were the rabbits.

OBSERVATIONS

The average distance between nearest neighbors in each of four quadrants of the circle of observed activity of each rabbit was found to be more suitable for measuring the relationship between ectoparasite populations than was the single "next-nearest neighbor method;" the latter frequently failed to measure certain spaces which affected tick populations. When, for example, hosts occurred over a narrow U-shaped or V-shaped area, as in converging brush-covered channels, and when the measurement of distances between centers of activity was begun at the top of one branch, the flow of measurements automatically proceeded down that branch and up the other without taking into account the space between them. When the hosts are distributed along narrow habitat one after another somewhat like beads on a necklace and the branches of the habitat are close, there must be multiple exchange of ectoparasites; but when far apart, relatively simple exchange. The nearest single-neighbor method is adequate only when habitat is uniform in all directions or where the branches are so wide apart that there is little or no exchange of parasites.

Dog ticks occurred on only 13 rabbits; 6 on area A and 7 on the other two areas. The habitat apparently was unsuitable for them. The number of bird and rabbit ticks on rabbits whose four neighbors were an average of from 2 to 40 yards distant varied from 0 to 19.7 times the base average number per rabbit. The mean was 3.7 times the base average. One rabbit (4 percent)

bore no ticks.

The number on rabbits whose neighbors were from 41 to 100 yards distant varied from 0 to 7.2 times the base average. The mean was 1.4 times the base. Five rabbits (22 percent) bore no ticks.

The number on those from 101 to 164 yards varied between 0 and 1.8 times the base. Nine (40 percent) bore no ticks. The mean for the group was 0.2 times the base.

Six (50 percent) of the rabbits whose neighbors were from 165 to 211 yards distant bore no ticks. The greatest infestation was 0.3 times the base.

A higher percentage (62) of rabbits was retrapped in the compact, stable habitat (A) than in the unstable habitats (B and II) where only 14 percent were retrapped.

SUMMARY AND CONCLUSIONS

A comparative analysis was made of data concerning the relation of spacing in rabbit populations and infestations by the bird and rabbit ticks on rabbits which were live-trapped, marked and released for subsequent observation.

Centers of activity of each rabbit were calculated by the method recommended by Hayne (1949), and distance between centers by a modification of the method recommended by Dice (1952). The average distance was measured from each center of observed activity to next nearest center in each of four quadrants except where, as at the margin of the study area, no observations were available. In the latter case the nearest unused distance in any quadrant was substituted.

On the compact stable habitat, where 109 rabbits were caught, 62 percent were caught more often than once each. On the other two areas, where stable rabbit habitat was widely interspersed among variable, unsuitable habitat, only 14 percent were caught more often than once each. It is believed that most of the rabbits on the latter areas were transients.

The relationship of tick infestation to average distance between rabbits was such that on semi-log paper a straight-line decrease is indicated, from an average infestation of 3.7 times as many ticks per rabbit as the base average for the county when rabbits averaged from 2 to 35 yards apart, to 0.2 times the base when rabbits averaged from 101 to 153 yards apart. The rate of decline in infestation seemed to level off when the average distance between hosts was more than 153 yards where, for 9 rabbits, the average infestation was 0.07 times the base.

The relatively large number of ticks on rabbits in dense populations and the relatively smaller numbers on members of sparse populations are believed to be related mostly to distance between hosts, particularly the rabbits.

However, the smaller tick populations also may be the result of death of the non-parasitic stages, lost when the rabbits spend time away from fence lines or suitable cover, in cultivated fields or other habitats not suitable to the ticks.

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PASSERINE BIRDS AS EXPERIMENTAL HOSTS FOR *POSTHODIPLOSTOMUM MINIMUM* (TREMATODA: DIPLOSTOMIDAE)

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Posthodiplostomum minimum (MacCallum, 1921) Dubois, 1936, is a common strigeoid trematode parasitizing several species of herons. Metacercariae of this species, formerly known as *Neascus van cleavei* (Agersborg), occur in a wide variety of fresh-water fishes, where they invade the liver, spleen, opisthonephroi, mesenteries, and pericardium. Numerous life history studies on this species have been published, including those by Ferguson (1936, 1938, 1940, 1943), Hunter and Hunter (1940), J. H. Miller (1954), and Hoffman (1958).

Several attempts to employ birds other than herons as experimental hosts for *P. minimum* have been reported. Ferguson (1936, 1938) and J. H. Miller (1954) showed that newly hatched, unfed chicks serve as suitable definitive hosts. Pigeons and ducklings, however, as well as chicks older than 4 days or those having been fed prior to exposure to metacercariae, remained refractory to infection, according to the latter investigator.

Adults of the 14 known species of *Posthodiplostomum*, according to Dubois (1957), demonstrate a marked specificity for birds of the Order Ciconiiformes, particularly herons (Family Ardeidae). Dubois believes that the selective mechanisms responsible for such specificity among avian strigeoids are related to chemical factors associated with the activity of the tribocytic organ (holdfast).

That host specificity among certain avian diplostomid flukes may be somewhat less rigid than Dubois maintains was demonstrated by a series of experiments involving metacercariae of *P. minimum*. A preliminary report of these studies (conducted during the summers of 1959 and 1960) at the Iowa Lakeside Laboratory at Lake Okoboji) was published in 1960.

FEEDING EXPERIMENTS

Great blue herons (*Ardea herodias*) of the Okoboji region carry heavy infections of *P. minimum*, whose metacercariae occur in several species of fish. Pumpkinseeds (*Lepomis gibbosus*) in Lake Okoboji show a very high rate of infection with metacercariae. Infected livers

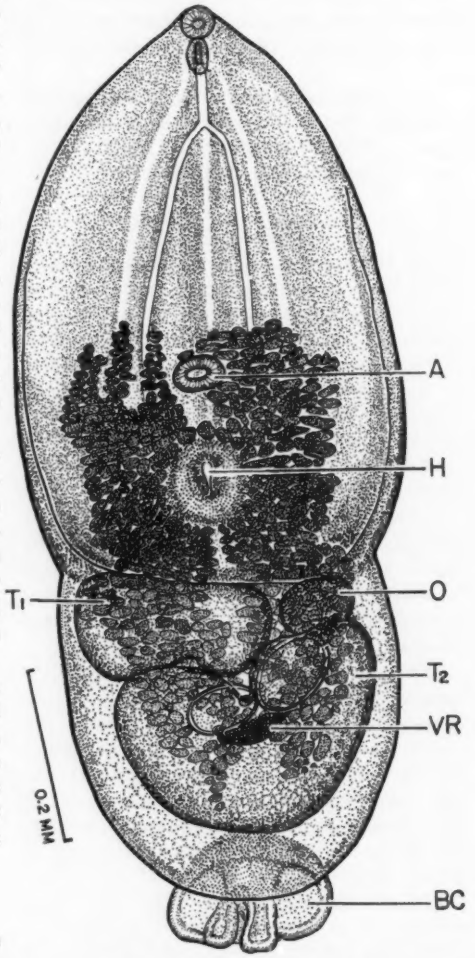

FIGURE 1. *Posthodiplostomum minimum* from young robin, 40 hour post-feeding. Abbreviations: A, acetabulum; BC, bursa copulatrix; H, holdfast; O, ovary; T_1, anterior testis; T_2, posterior testis; VR, vitelline reservoir.

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of these fish served as the source of metacercariae in all feeding experiments discussed below.

On June 25, 1959 two nestling robins (*Turdus migratorius*) were removed from their nest; one was force-fed the liver of a naturally infected pumpkinseed. Forty hours after feeding, 182 adult *P. minimum* were removed from the robin's intestine. The second bird, kept as a control, was negative for strigeoids when examined 40 hours after the experiment was begun. Examination of 97 whole mounts prepared from the flukes found in the first robin indicated that 47 adults (48 percent) were gravid.

A second experiment involving a nestling robin was conducted in June, 1960. Forty-eight hours after feeding an infected pumpkinseed liver to this host, 240 gravid and 22 immature *P. minimum* were recovered. In both experiments, gravid adults (fig. 1) corresponded in all respects to *P. minimum* recovered from naturally infected great blue herons of the region. Eggs of those worms reared in robins appeared normal in every way, but no attempt was made to follow their further development.

The results of these experiments involving robins suggested the feasibility of using other passerines as possible experimental hosts. During the summer of 1960, four red-winged blackbird nestlings (*Agelaius phoeniceus*) and two yellow-headed blackbird nestlings (*Xanthocephalus xanthocephalus*) were force-fed livers of infected *Lepomis gibbosus* containing large numbers of *P. minimum* metacercariae. No adult worms were recovered from the yellow-headed blackbirds 48 hours post-feeding. Two of the red-wings were examined 40 hours after feeding. Only immature worms (144 in one bird, 2 in the other) were recovered. The remaining two red-wings were examined 48 hours after feeding, but proved negative for adult flukes.

A summary of all feeding experiments is presented in table I.

TABLE I. Summary of feeding experiments.

Avian host	Date of feeding	Date of examination	Number of adult worms recovered		Age of worms
			Gravid	Immature	
Robin (*Turdus migratorius*)	6/25/59	6/27/59	47*	50*	40 hours
	6/27/60	6/29/60	240	22	48 hours
Red-winged blackbird (*Agelaius phoeniceus*)	6/15/60	6/17/60	0	144	40 hours
	6/24/60	6/26/60	0	2	40 hours
	6/15/60	6/17/60	0	0	48 hours
	6/24/60	6/26/60	0	0	48 hours
Yellow-headed blackbird (*Xanthocephalus xanthocephalus*)	6/21/60	6/23/60	0	0	48 hours
	6/21/60	6/23/60	0	0	48 hours

* Total number of worms recovered was 182. Figures represent only those specimens prepared as whole mounts.

DISCUSSION

The experimental evidence presented above is the first to demonstrate the suitability of certain passerine birds as experimental hosts for *P. minimum*.

The pronounced host specificity of adult diplostomid flukes is considered of value by Dubois (1944, 1955) in establishing taxonomic relationships among strigeoid trematodes. Baer (1951:166) suggested that host-specificity of trematodes in general is due to ". . . ecological segregation of the larval stages and also to selective feeding habits of the definitive host. . . ." But Dubois (1957:224–225) emphasized that host-specificity among strigeoids cannot be attributed merely to ecological segregation, but that such specificity involves additional factors. These relate in part to the nature of the tribocytic organ (holdfast) by means of which is established a much closer host-parasite association than that characterizing most trematodes. The ability of the strigeoid holdfast to serve as a nutritive structure has been indicated by several investigators including LaRue (1927), van Haitsma (1931), Szidat (1929), and Ulmer (1955). Thus, an even greater degree of intimacy between the adult worm and the host mucosa is established. Host specificity of strigeoids, therefore, according to Dubois (1957), is a consequence of the evolution of both parasite and host. He concludes " . . . L'évolution des Strigeides . . . semble donc marquée par la

tendance générale (plus ou moins manifeste selon les groupes progressivement ségrégés) de remplacer la spécificité écologique par la spécificité phylogénique. C'est pourquoi il est possible d'établir pour ces Helminthes une *systématique biologique* fondée essentiellement sur la combinaison des caractères morphologiques dont il vient d'être question et des données statistiques relatives à la spécificité parasitaire. . . ."

Apparently there is need for additional experimental data relative to host specificity in order to assess the validity of Dubois' use of it as a major criterion for establishing taxonomic relationships. Obviously, ecological segregation and selective feeding habits play an important role. Additional experimental exposures of avian strigeoid metacercariae to diverse groups of birds might contribute much to our understanding of these relationships.

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PARALLELORCHIS DIGLOSSUS N. G., N. SP., A TREMATODE (STRIGEIDA: DIPLOSTOMIDAE) FROM THE FLORIDA RACCOON* **

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Five raccoons, *Procyon lotor*, out of 19 collected in Glades County, Florida, in August 1960, were found to harbor specimens of an unknown strigeoid trematode in the small intestine. This form possesses a prominent genital atrial recess and a bi-lobed tribocytic organ, characteristics which warrant the erection of a new genus. We assign this genus to the family Diplostomidae, sub-family Alariinae. The name *Parallelorchis diglossus* is proposed for this trematode. The generic name indicates the parallel position of the testes in the equatorial zone of the body and the specific name refers to the very prominent bi-lobed tongue-shaped tribocytic organ.

Parallelorchis n. g.

Generic diagnosis: Family Diplostomidae: sub-family Alariinae. Body vase-shaped, robust, approximately straight with no clear demarcation between forebody and hindbody, forebody wider, hindbody subcylindrical. Tribocytic organ consisting of two lateral tongue-like lobes, conspicuous, often extending beyond anterior end; arising from forebody without any posterior demarcation. Pseudosuckers present. Vitellaria, follicular, restricted to forebody. Ovary at level of anterior third of body. Mehlis' gland lateral and posterior to ovary, anterior to left testis. Testes, ovoid, approximately equal; smooth in outline, usually situated side by side in midbody. Bursa well-developed with conspicuous ventral papilla and atrial recess with glands (presumably prostatic); ejaculatory pouch absent.

Type species: *Parallelorchis diglossus* n. sp.

Parallelorchis diglossus n. sp.
(Figs. 1–5)

Description: (Measurements in mm, obtained from 22 specimens.) Body robust, vase-shaped, (length 2.54 to 3.40) covered with minute spines; anterior portion resembling a rolled tongue, incorporating an anteriorly projecting bi-lobed tribocytic organ; body gradually tapering behind testes but with no definite demarcation between forebody and hindbody. Forebody slightly longer and much wider (1.09 to 1.56 at level of acetabulum) than hindbody, gradually tapering posteriorly to the subcylindrical hindbody (0.707 to 1.090 wide). Posterior portions of lateral body folds of forebody almost contiguous in mid- ventral line. Anterior end of forebody sometimes reflexed slightly dorsad. Pseudosuckers present, invaginated, weakly glandular. Weakly developed oral sucker (0.095 to 0.142 by 0.132 to 0.165) terminal, at tip of terminal cone (similar to that of *Fasciola hepatica*). Acetabulum (0.082 to 0.122 by 0.095 to 0.129) not raised above body surface, situated between base of tribocytic organ and forebody, completely covered by tribocytic organ. Latter well developed, consisting of two tongue-like projections in juxtaposition; appearing as extension of ventral wall of forebody without separating constriction from body proper; in many specimens extending anteriorly beyond edge of oral sucker; base of organ with prominent glands. Prepharynx absent, pharynx (0.102 to 0.136 by 0.102 to 0.122) moderately well developed; esophagus very short, ceca extending nearly to posterior end, passing ventrad to testes. Vitellaria follicular, confined to forebody and concentrated in two lateral fields, absent from tribocytic organ; dorsal vitellaria in two triangular fields in extreme lateral areas of forebody; ventral vitellaria in lateral folds and extending medially on each side of ovary. Vitelline ducts originating laterally from posterior portion of vitellaria, proceeding medially to vitelline reservoir; latter sac-like, dorso-ventrally elongated with short duct emptying into oviduct.

Testes 2, large, elongate-ovoid, smooth, situated side by side in midbody, separated by vas deferens and descending limb of uterus; either testis slightly anterior to other. (Left testis 0.476 to 0.707 by 0.308 to 0.576, right testis 0.476 to 0.707 by 0.313 to 0.576.) Vasa efferentia arising from anterior ventro-medial surface of respective testes, proceeding anteriorly and emptying separately into highly convoluted seminal reservoir. Latter immediately anterior to testes, more or less median, size variable. Vas deferens sinuous, arising from posterio-median portion of seminal reservoir, proceeding posteriorly between testes to closely convoluted seminal vesicle, latter immediately posterior to testes. Seminal vesicle distended with masses of

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sperm. Ejaculatory duct weakly muscular, arising from dorso-posterior surface of seminal vesicle, curving ventrally and then posteriorly to open into very short hermaphroditic duct dorsal to entrance of uterus.

Ovary reniform (0.163 to 0.204 by 0.218 to 0.354) transversely elongated, situated medio-dorsally in forebody. Oviduct arising from dorso-posterior surface of ovary, proceeding posterio-laterally, receiving common vitelline duct just before entrance into anterior end of Mehlis' gland; latter usually anterior and medial to left testis but situs inversus demonstrated in some specimens. Laurer's canal present. Oviduct S-shaped in Mehlis' gland, ootype posterior, uterus arising at posterior end of Mehlis' gland and immediately curving anteriorly and proceeding as convoluted tube to level of pharynx, barely entering base of tribocytic organ; proximal portion of ascending limb often filled with sperm. Descending limb of uterus often distended by numerous eggs; passing posteriad in mid-ventral plane between testes, curving dorsally in posterior region of hindbody to empty into hermaphroditic duct. Latter emptying into bursal atrium short distance from posterior end.

Bursa bell-shaped with anteriorly directed narrow recess terminating in spherical space bounded by muscular walls. Secretion (presumably prostatic) visible in sections of entire wall of bursa and recess anterior to genital pore. Parenchyma of middle area of hindbody compact, without excretory spaces, consequently more heavily stained than peripheral areas; numerous nuclei present in parenchyma, indicative of presence of prostatic glands. Bursa containing well developed papilla subterminally on its ventral surface; bursal cavity opening dorso-terminally by large orifice.

Eggs operculated (0.089 to 0.115 by 0.063 to 0.083), very numerous.

Host: Procyon lotor.

Habitat: Small intestine.

Locality: Glades County, Florida.

Specimens: U. S. N. M. Helm. Coll. No. 39073 type, No. 39074 paratypes, including sagittal sections.

There are three additional genera of strigeoids which have the testes situated side by side: *Pharyngostomum* Ciurea, 1922, *Pharyngostomoides* Harkema, 1942, and *Procyotrema* Harkema and Miller, 1959. These are parasites of carnivores and the last two are found in the raccoon. *Parallelorchis* can be separated from all three genera by the morphology of its tribocytic organ which is not separated from the body and consists of two lateral tongue-like lobes. *Pharyngostomum* also has an atrial recess but it does not terminate in a spherical space as does that of *Parallelorchis*. Furthermore, LaRue (1926) states: "There is no indication of gland cells around this structure (in *Pharyngostomum*) but there are well-developed muscle fibers." *Pharyngostomoides* possesses an ejaculatory pouch which is absent in *Parallelorchis*. The latter does not possess the ventral pouch characteristic of *Procyotrema*.

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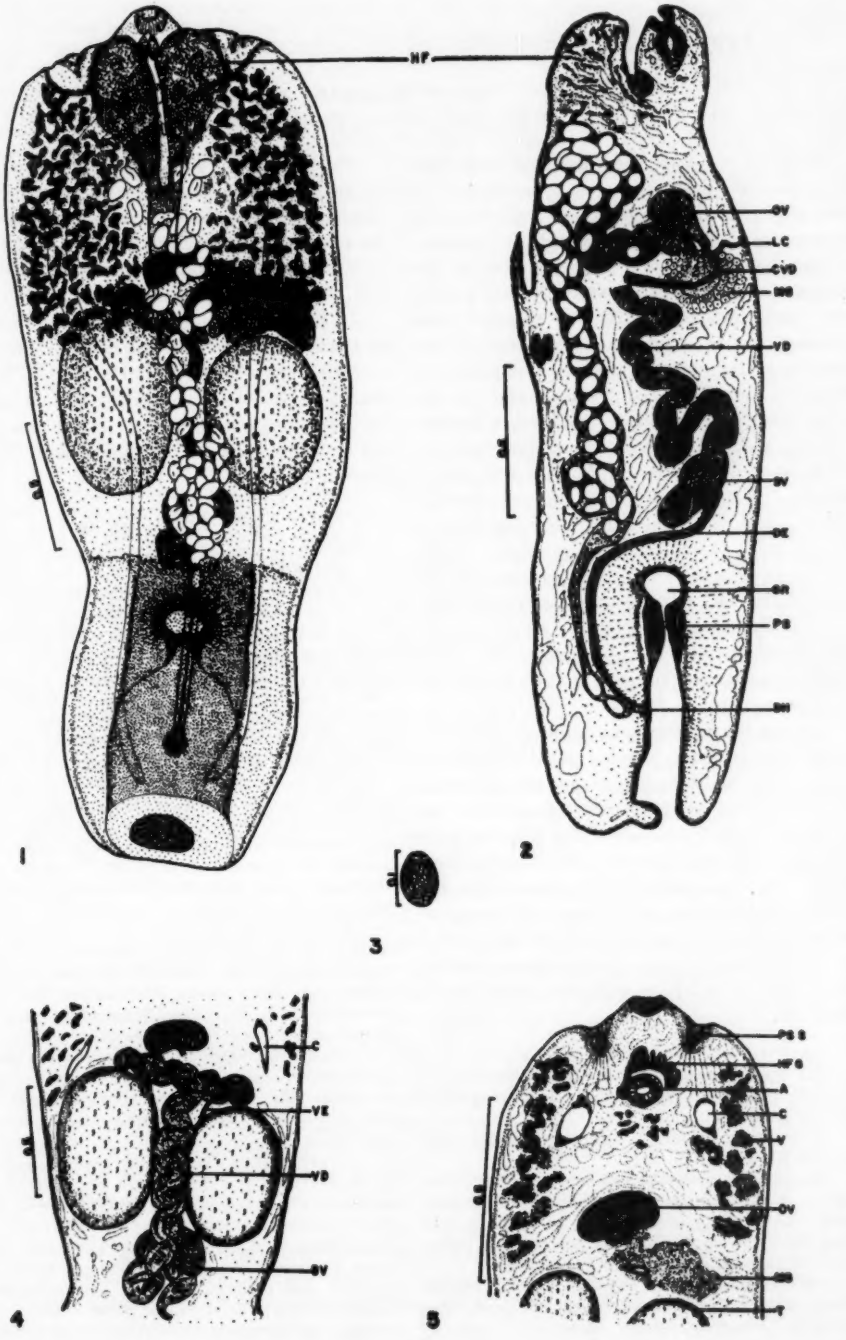
EXPLANATION OF PLATE

Abbreviations: A, acetabulum; C, caecum; CVD, common vitelline duct; DE, ductus ejaculatorus; DH, ductus hermaphroditicus; GR, genital recess; HF, holdfast organ (tribocytic organ); HFG, holdfast gland; LC, Laurers' canal; MG, Mehlis' gland; OV, ovary; PS, glandular secretion; PSS, pseudosucker; SV, seminal vesicle; T, testis; V, vitellaria; VD, vas deferens; VE, vas efferens. (Scales in mm.)

FIGURE 1. Whole mount, ventral view.
FIGURE 2. Sagittal section of entire worm, partially reconstructed.
FIGURE 3. Operculated ovum.
FIGURE 4. Frontal section, level of testes.
FIGURE 5. Frontal section, anterior end.



1 2 3 4 5

PAPILLAE ON THREE SPECIES OF SCHISTOSOME CERCARIAE*

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Papillae in or on the cuticle of trematode cercariae have been reported in a number of distantly related groups. Some authors have referred to these structures as sensory papillae or papillae with sensory "hairs" or bristles. Although a sensory function seems probable, such has been inferred only on morphological grounds. No function other than sensory has been suggested. Among reports of papillae are those by Stunkard and Shaw (1931) on an echinostome; Ito (1956a,b; 1957) on a cyathocotylid, a long tailed opisthorchioid and an echinostome; Fischthal (1951) on five gorgoderids; Coil (1954, 1955) on two gorgoderids; Cable (1953, 1956) on fellodistomatids, echinostomes and opecoelids (He pictured similar structures on cercariae in other groups, but did not discuss them in the text.); Cable and Hunninen (1942) on a lepocreadiid; Allison (1943) and Ulmer (1951) on brachylaemids; Wall (1941) on a spirorchid; and Seitner (1951) on an alloecreadiid.

Among the schistosomes, papillae have been noted several times. Gordon et al (1943) reported them on *Schistosoma haematobium* and *S. mansoni* but pictured only those on the tail of the cercaria. They stated that papillae were present on the body, but that no definite pattern could be discerned. Vercammen-Granjean (1951) noted similar structures on *S. mansoni;* he drew only those on the body. Szidat (1951) observed two pairs on a schistosome cercaria, for which the adult is unknown, from South America.

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Gordon et al (1934) stained the papillae on *S. haematobium* and *S. mansoni* with carbol fuschin and said that the stain could be made permanent. Seitner (1951) used silver nitrate to stain these structures on *Allocreadium ictaluri.*

In the present work the number and position of the papillae on three species of schistosome cercariae are described, and a method is presented for staining these structures and preparing permanent whole mounts. An abstract dealing with this work has been published (Wagner, 1959).

MATERIALS AND METHODS

Cercariae studied were those of *Schistosomatium douthitti* (Cort, 1914) Price, 1931; *Schistosoma mansoni* Sambon, 1907; and *Ornithobilharzia canaliculata* (Rudolphi, 1819) Odhner 1912.

S. douthitti and *S. mansoni* cercariae were secured from laboratory raised and infected *Lymnea palustris* and *Australorbis glabratus* respectively. The original stock of infected *L. palustris* was obtained near Ann Arbor, Washtenaw County, Michigan, and was replenished with infected snails collected in Cheboygan County, Michigan. The *S. mansoni* and *A. glabratus,* of Puerto Rican origin, were obtained from Dr. M. A. Stirewalt at the National Naval Medical Center, Bethesda, Maryland. Snails were maintained according to the techniques described by Lee and Lewert (1956), with the exception that water from the Wakulla River, Florida, was used instead of treated tap water.

Batillaria minima, a marine gastropod, served as intermediate host for *O. canaliculata.* Cercariae were derived from naturally infected specimens collected at Piney Point, Florida, and kindly furnished by Dr. L. R. Penner. These snails were maintained as described by Wagner (1960).

Adult specimens of *O. canaliculata,* derived from the cercarial stock used, have been deposited in the Florida State University parasite collection (Nos. 279–296). These worms were reared in pigeons.

In preparation for staining papillae, cercariae of *S. douthitti* and *S. mansoni* were placed on a slide in a small drop of water and fixed with fumes from dry osmium tetroxide crystals or from a solution containing 4 parts 2 percent osmic acid and 15 parts 1 percent chromic acid. The water was then removed and replaced with 0.5 percent silver nitrate solution. The cercariae were allowed to remain in the silver nitrate solution for 10 minutes in

the dark after which they were washed with several changes of distilled water and then usually placed in sunlight or artificial light for from 5 to 10 minutes. With artificial light it was possible to selectively darken the papillae in a particular area by placing the slide on the microscope stage and closing the field and condenser diaphragms so that only a small part of the cercaria was illuminated.

Considerable difficulty was experienced in staining the papillae of *O. canaliculata*. There was a tendency for silver chloride, which turned brown on exposure to light, to precipitate on the cuticle, an effect which apparently resulted from the marine water in which the cercariae are shed. This difficulty was to some extent overcome by fixing cercariae as soon as possible after they had emerged from the snail, washing them in several changes of distilled water, and then placing them in 5 percent potassium nitrate (Harmer, 1884) for 1 hour. Even with these precautions some staining of the cuticle occurred, and the results were more difficult to interpret than in the fresh water species.

Permanent mounts were made of stained cercariae in 10 percent glycerine solution. The slides were coverslipped but not ringed. As the preparation dried glycerine was added to prevent excessive flattening of the cercariae.

RESULTS

Papillae were observed on cercariae of all three species. They appeared to be of several types and, on living cercariae, a seta was seen on each papilla. In unstained specimens the papillae appeared as small, rounded elevations on the integument. Generally they could be seen only in profile, when the cercaria assumed a favorable position. In stained preparations the individual papillae appear as dark spots, sometimes bordered by a darker ring, on an unstained background. They appear to be small areas where the cuticle of the cercariae is absent with the underlying cells protruding to form the elevations. The setae associated with the papillae are usually destroyed during staining, but are visible on fixed, unstained specimens.

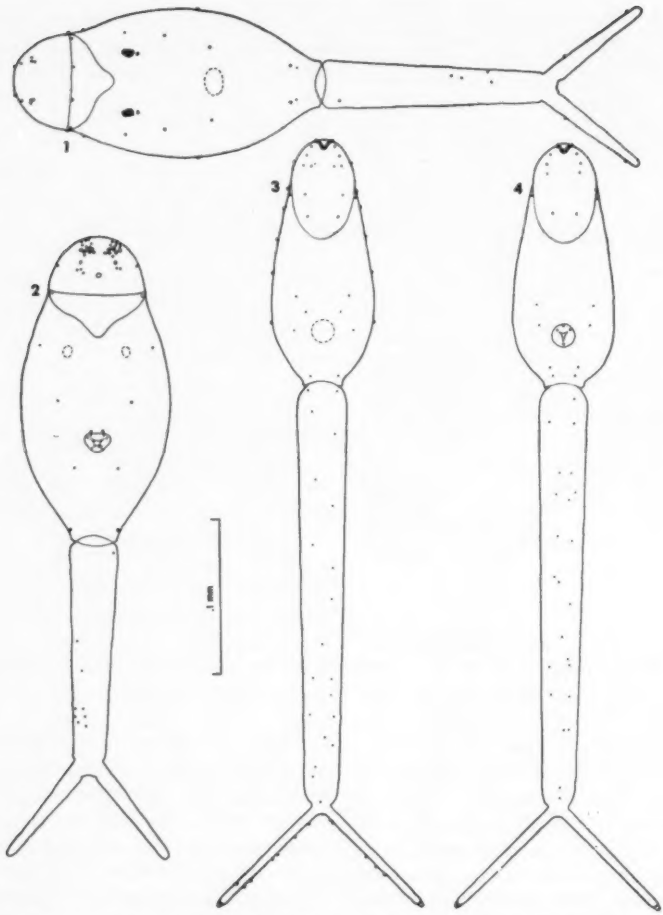
On cercariae of all three species there is a group of papillae mounted on stalks at the anterior end of the head organ. These have been referred to as "penetration spines" by various authors. Due to their staining reaction, and morphology, it is believed that their function is similar to that of the other papillae on the body and tail.

Aside from the papillae, other structures stained by the silver nitrate technique are: the mouth opening, openings of the penetration gland ducts, and the excretory pores at the tips of the furcae.

The following accounts are based on about 20 stained specimens of each species. On the cercarial body the pattern in each of the three species presented a basic bilateral symmetry which was fairly regular, but not absolutely constant. On the tail greater irregularity occurred. Papillae on the tail stem of *S. mansoni* are located dorsally and ventrally with none laterally. It is believed that this is also true of *S. douthitti* and *O. canaliculata*, but this point could not be determined with certainty. On the furcae of these two species the pattern was relatively constant, whereas on *S. mansoni* there was some irregularity.

Schistosomatium douthitti (figs. 1 and 2). In living specimens the papillae on the body are about 2 microns in diameter; a seta about 7 microns long was seen on each papilla which was favorable for study. The papillae at the anterior end ("penetration spines") are about the same size, but have much shorter setae, about 1 or 2 microns in length. On the tail stem the papillae are smaller but have setae of about 10 microns. After staining, two pairs of papillae on the body appeared considerably larger than the others, one pair immediately behind the eye spots, the other near the posteroventral edges. Neither of these pairs was seen in living specimens nor were setae ever observed on them. On stained cercariae, six papillae located on the acetabulum and one posterodorsal pair appeared smaller than the other body papillae. These also were not observed in living specimens. The anterior end of the specimen drawn (fig. 2) was somewhat bent so that the openings of the gland ducts and the anterior papillae appear from a ventral view. On this specimen one of the papillae lateral to the mouth opening is unpaired and an extra papilla is present on the opposite side, in the group near the anterior end.

Schistosoma mansoni (figs. 3 and 4). The papillae on this species are slightly larger than those on *S. douthitti*, all appearing to be about 3 microns in diameter. The setae are about 10 microns long on all the papillae except those on the acetabulum and the group at the anterior end which have setae 1 or 2 microns in length. After fixation a small depression remained at the anterior end containing the openings of the penetration gland ducts and the anterior group of papillae. The stainable structures in this area were so crowded that individual papillae



Figures 1–4. Cercariae, showing papillae stained with silver nitrate. (Figures drawn with the aid of camera lucida.) 1, *Schistosomatium douthitti*, dorsal and lateral papillae; 2, *Schistosomatium douthitti*, ventral papillae. (Mouth and gland duct openings shown as circles.); 3, *Schistosoma mansoni*, dorsal and lateral papillae; 4, *Schistosoma mansoma*, ventral papillae.

and gland openings could not be discerned. The number and position of the papillae on the tail furcae was quite variable, but they always occurred on the posterior surface of the furcae.

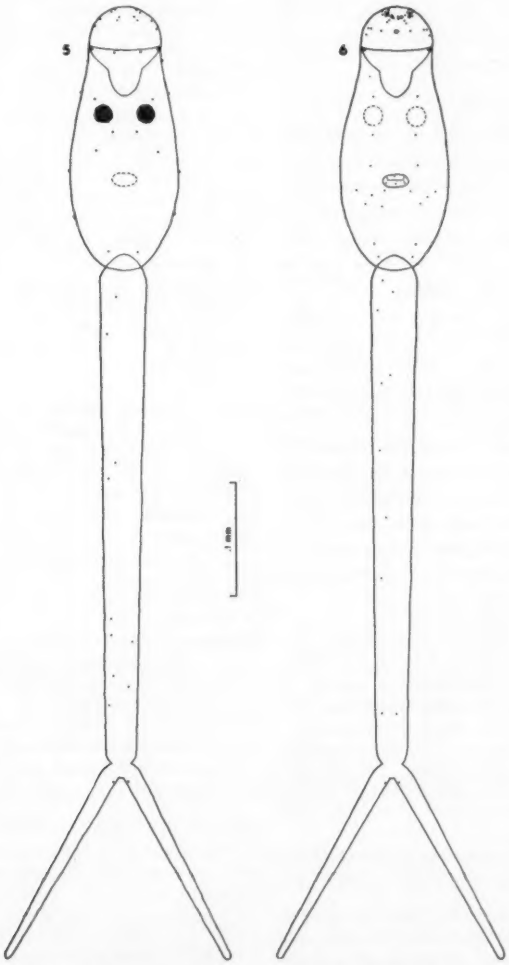
Ornithobilharzia canaliculata (figs. 5 and 6). The figures were drawn from two cercariae, the head organ and tail of one and the body proper of another. Except for the "penetration spines," papillae and setae on the body and tail could not be seen well or measured on live specimens. On living cercariae the "penetration spine" papillae were about 2 microns in diameter with setae 1 or 2 microns in length. After staining, all of the papillae appeared about the same size except for those on the acetabulum which were somewhat smaller. Only six gland duct openings could be discerned on the head organ. It seems probable that six more were present; but either they were not stained or, more likely, could not be differentiated from papillae.

DISCUSSION

Except for the "penetration spines" papillae have not been reported on the cercariae of *S. douthitti* or *O. canaliculata*. On *S. mansoni*, however, they have been described by Vercammen-Granjean (1951). He stated that the pattern on the body is constant and symmetrical,

with 24 papillae on the body including the head organ; he noted one pair of papillae without setae. The "penetration spines" were figured, but he did not mention them in the text, nor did he figure or mention papillae on the acetabulum. In contrast, during the present investigation, some variation in number and distribution of papillae was noted; 53 papillae, exclusive of the "penetration spines," were seen on the body and head organ of the cercaria illustrated (figs. 3 and 4), and four of these were on the acetabulum. Although this disagreement probably is due, at least in part, to different methods of observation, there is a possibility that papillar patterns differ among strains of the parasite. Vercammen-Granjean did his work in the Belgian Congo, presumably on local material, whereas the original source of the material used for the present study was Puerto Rico. Another difference is in the intermediate hosts. Vercammen-Granjean used *Biomphalaria* (*Planorbis*) *stanleyi* while *Australorbis glabratus* was used in the present study.

The use of cercarial papillae as taxonomic



Figures 5 and 6. Cercariae, showing papillae stained with silver nitrate. (Figures drawn with aid of camera lucida.) 5, *Ornithobilharzia canaliculata*, dorsal and lateral papillae; 6, *Ornithobilharzia canaliculata*, ventral papillae; mouth and gland duct openings shown as circles.

characters has been suggested by Vercammen-Granjean (1951) and Kuntz (1955). The present author further suggests that their study may aid in identification of cercariae without characteristic behaviour patterns or distinctive morphological characters which are readily observable. It also seems possible that permanent mounts of cercariae prepared in the manner outlined may be useful as type specimens when the adult stage is unknown.

It would be interesting to determine whether or not infraspecific variation in papillar pattern has a genetic basis; for if it does, papillae might prove useful in distinguishing sex of schistosome cercariae as well as in various types of genetic studies on trematodes.

SUMMARY

Surface papillae were studied on cercariae of *Schistosoma mansoni*, *Schistosomatium douthitti*, and *Ornithobilharzia canaliculata* after fixation by osmium tetroxide fumes and subsequent staining by silver nitrate impregation. Permanent whole mounts in glycerine can be made after staining in this manner.

In each of the three species there is some variation in number and position of papillae. A fairly regular, bilaterally symmetrical pattern occurs on the body; on the tail there is greater variability. Structures at the anterior end of the cercariae, which have been described as "penetration spines" are stained by this procedure and are believed to be analogous to the other papillae on the body and tail. Most, if not all, of the papillae bear a seta which is usually destroyed during the staining procedure.

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HETEROBILHARZIA INFECTION IN THE DOG AND OTHER MAMMALS IN LOUISIANA*

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Severe dermatitis apparently acquired from wading in the swamps of Southern Louisiana has been observed among trappers, oil field workers and others (Burks and Jung, 1960). In an attempt to determine the possible role of zoonotic schistosomiasis in this disease, examinations were made of snails, birds and mammals. One species of schistosome, *Heterobilharzia americana* Price, 1929, was found in the raccoon (*Procyon lotor*), the nutria (*Myocaster coypus*), and the dog. *H. americana* previously has been recorded from a bobcat (*Lynx rufus*) in Florida (Price, 1929) and from raccoons in Texas (Price, 1943), North Carolina (Miller and Harkema, 1960), and Florida (W. H. Leigh, University of Miami, personal communication, 1960).

OBSERVATIONS

In the present study *H. americana* was found in 25 of 52 racoons, 22 of 37 nutrias, and 3 of 3 dogs examined from the Mississippi River delta and marshy coastal areas of Louisana (fig. 1). At Pass à Loutre, the infection was found in 21 of 39 raccoons, 19 of 24 nutrias, and each of the three dogs living in that area (2 by fecal examination); 2 of 7 raccoons and 3 of 5 nutrias at Lafitte, and 2 of 3 raccoons at Avery Island were infected. Three raccoons and 8 nutrias taken at Marsh Island were uninfected. The infected nutrias and raccoons from Pass à Loutre were examined during the months of February, April, May and June. As all young mature nutrias, about 5 months old, were uninfected, transmission of infection appears to be seasonal. Infection among the snail intermediate hosts is apparently seasonal also (Lee, 1960). The number of worms recovered from the nutrias varied from 15 to 56 and from the raccoon, 4 to 186. From 1 dog 87 pairs in copula and 15 male worms were collected.

FIGURE 1. Map of Louisiana indicating localities where mammals infected with *Heterobilharzia americana* were collected.

In all three host species, the worms were located in the small veins of both small and large intestines, and in the intrahepatic portal veins. One of three swamp rabbits, *Sylvilagus aquaticus*, at Pass à Loutre was also found infected.

The dog, which was examined post mortem, was a 6-year-old mongrel which often waded or swam in the swamps of the wildlife refuge at Pass à Loutre. Progressive emaciation along with intermittent dysentery and an eczematoid condition had been observed for about a year. When first seen by us in the refuge, he was in extremely poor condition with a halting gait and feeble reflexes. When brought to the laboratory a week later, he had severe purulent conjunctivitis, no appetite, bloody diarrhea, and the stools contained masses of *H. americana* eggs; evidences of other parasites were not observed. The following day he became comatose and was sacrificed.

The mucosal surface of the entire intestine contained scattered small petechiae and, in some

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areas, there were accumulations of bloody mucus. The liver was slightly enlarged, pale in color with diffuse yellowish spots 1 to 2 mm in diameter on its outer and cut surfaces. A number of adult schistosomes extruded from the cut surfaces. The spleen was slightly enlarged and slate-gray in color. The kidneys and pancreas appeared to be normal. Small hemorrhages were present in some areas of the lungs.

In sections of the intestine, schistosome eggs were present in all layers, but were most abundant in the submucosa, and occurred usually

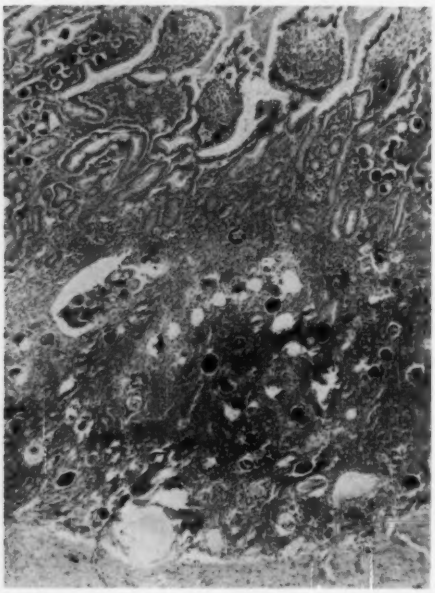

FIGURE 2. Eggs of *Heterobilharzia americana* in the submucosa and mucosa of the small intestine of dog . × 40.

in groups (fig. 2). In many areas the submucosa was almost obliterated by large masses of eggs (fig. 3). Eggs were numerous in the muscularis layer, often just beneath the serosa, but no peritonitis was evident. The associated inflammation was chiefly chronic with a dense infiltration of round cells, mainly in the submucosa. Eosinophils were few. Granulomata were uncommon and were generally limited to the muscularis.

In sections of the liver eggs were numerous along the portal triads and in the parenchyma. Pseudotubercle formation around the eggs was common (figs. 4, 5). Some portal triads were replaced by fibrous tissue surrounding the rem-

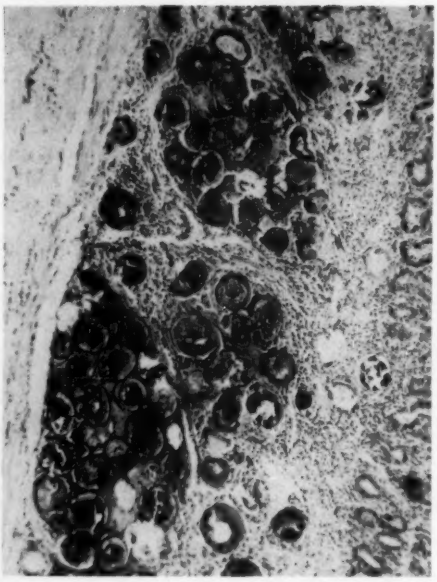

FIGURE 3. Nests of *Heterobilharzia americana* eggs in submucosa of small intestine of dog. × 100.

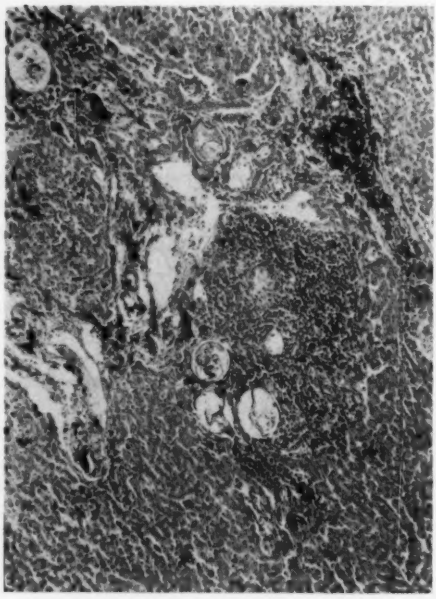

FIGURE 4. Pigment deposits and pseudotubercles around eggs of *Heterobilharzia americana* in liver of dog. × 100.

nants of eggs. Heavy deposits of pigment were scattered throughout the liver. Similar pigment deposits were present in the spleen where no schistosome eggs nor granulomata were observed. A few eggs were scattered throughout the kidney cortex and the lungs.

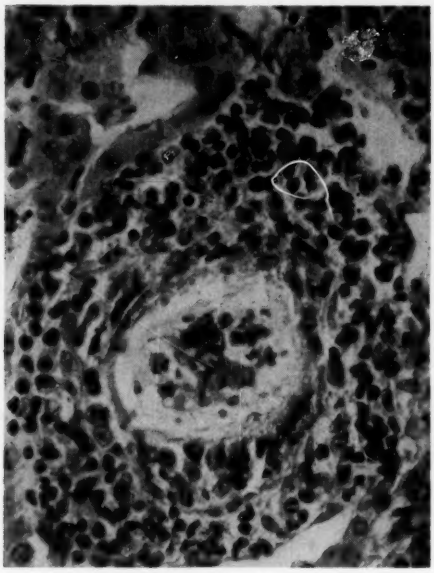

FIGURE 5. An enlarged pseudotubercle containing an egg of *Heterobilharzia americana* in liver of dog. × 446.

DISCUSSION

Observations indicate a wide range of hosts for *H. americana*: the nutria, a rodent; the raccoon, a carnivore (Procyonidae); and the dog, a carnivore (Canidae). In all these mammals the host-parasite relationship appeared to be well established as evidenced by the large number of viable eggs discharged with the stools and the numerous worms recovered at autopsy. Pronounced symptoms were associated with an advanced, heavy infection in the dog, but these symptoms were not observed in two other dogs with moderate or light infection. The wide range of hosts for *H. americana* becomes especially striking when compared with that of *Schistosomatium douthitti* (Cort, 1914) H. F. Price 1929, the only other known mammalian schistosome in North America.

The heterobilharziasis found in dogs in the Mississippi delta is the first canine schistosomiasis recorded for the Western Hemisphere. In some parts of the southern states it may prove to be an important disease of dogs. In other parts of the world, other species of schistosomes that naturally occur in dogs are: *Schistosoma japonicum* Katsurada, 1904 (Orient); *S. rodhaini* Brumpt, 1931 (Belgian Congo); *S. suis* Rao and Ayyar, 1933, which possibly is identical with *S. incognitum* Chandler, 1926 (India); and *S. spindalis* Montgomery, 1906 (India). Attempts by several workers to infect dogs with *S. mansoni* Sambon, 1907, were unsuccessful. Kuntz (1955) reported that the dog and carnivores in general are poor, almost incompatible, hosts for *S. mansoni* and for *S. haematobium*. From the liver of 1 of 11 dogs, Kuntz et al (1953) recovered small immature *S. mansoni* 5 weeks after exposure to thousands of cercariae. One successful trial with *S. mansoni*, in which viable eggs were recovered from the feces of two pups 79 to 81 days after exposure to cercariae, was reported by Pinto and Almeida (1945). In the case of *Schistosomatium douthitti*, cercariae have been found naturally and experimentally to cause only dermatitis in dogs (Herber, 1938).

H. americana also occurs naturally in feline hosts (*Lynx rufus*), as do *S. japonicum*, in domestic cats in the Orient, and *S. rodhaini*, in the serval (*Felis serval*) of the Belgian Congo (Fain et al, 1953).

Thus, *H. americana* is similar to *S. japonicum* and *S. rodhaini* in its adaptability to rodents, canines, and felines. D'Haenens and Santele (1955) recovered eggs of *S. rodhaini* from the stools of natives in Elizabethville in the Belgian Congo. The extent to which *H. americana* would develop in man is not known, but the cercariae probably would penetrate the skin and the schistosomulae possibly would invade the lungs and the liver, at least to a greater extent than would those of *S. douthitti*, the only other species of schistosome known to occur naturally in mammals of North America.

Heterobilharziasis appears to be highly enzootic in the coastal swamplands of Louisiana and the mud flats of the Mississippi River delta where conditions are ideal for transmission. Both the nutria and the raccoon frequent these habitats and are good swimmers. The nutria is essentially an aquatic animal and much of the time is submerged in the water. The large populations of raccoons with high mira-

cidia-producing capacity, apparently maintain high levels of infection in the snails of these localities. Thus, there is heavy infestation of the waters with cercariae which may be a cause of the dermatitis acquired by persons frequenting the swamps. Although its egg-passing capacity has not been established, the possible significance of the nutria in the epizootiology of mammalian schistosomiasis is apparent in view of its wide distribution and abundance in many parts of Louisiana, Florida, New Mexico, Kansas, Nebraska, and some northwestern states. It was first introduced and became established in Louisiana about 1937 and is said to be spreading rapidly at the expense of the muskrat (*Ondatra*).

The pathological picture of *H. americana* infection in the dog confirms the general view that the ova play an important part in the pathogenesis of schistosomiasis. The eggs were formed in localized lesions of varying severity in the liver, lungs, intestines, and kidneys. These lesions were similar to those produced by other species of schistosomes, viz, *S. rodhaini* (Thienpont et al, 1953), *S. mansoni* var. *rodentorum* and *S. rodhaini* (Schwetz, 1956), *S. haematobium, S. mansoni* and *S. japonicum* (Meleney et al, 1952, 1953) and *Schistosomatium douthitti* (Kagan and Meranze, 1957).

Among the manifestations of intestinal schistosomiasis, especially that caused by *S. mansoni*, is enlargement of the liver and spleen. The pathogenesis of the enlarged liver is a matter of dispute. The liver of the dog infected with *Heterobilharzia* contained many granulomata, but was only slightly enlarged and cirrhosis was not evident. Several workers have reported similar findings on other species of schistosomes. Examination of additional mammals infected with *Heterobilharzia* will be necessary to provide a more complete knowledge of the nature and range of pathological conditions produced by this schistosome.

SUMMARY

Heterobilharzia americana was commonly found in raccoons and nutria from three localities in southern Louisana and in all of three dogs examined from one area. Infection in one of the dogs was fatal. This is the first record of canine schistosomiasis in the Western Hemisphere.

In all four host species, the worms were located in the veins of both small and large intestines and in the intrahepatic portal veins. Eggs were abundant in the wall of the intestines, and granulomata containing eggs were conspicuous in all parts of the liver. In the dog, eggs were also found in the lungs, kidneys and spleen.

ACKNOWLEDGMENT

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RESEARCH NOTE

A SIMPLE PRISMATIC DEVICE FOR VIEWING NEMATODE PARASITES IN VITRO CULTURES.

In axenic cultivation of nematode parasites, periodic observation of the culture with some magnification and without contamination is often desirable. Generally, the parasites are beneath several millimeters of liquid media in a glass container. Thus, for example, in viewing a tube culture microscopically, one must penetrate optically the glass, the space within, and the media before observing the parasites at the bottom.

An inversion system is effective in observing parasites on the bottom of a vessel. However, the rapid serpentine movements of nematode larvae, especially when exposed to intense light during magnification, make it difficult to keep the specimens within the limited field of a compound microscope. A stereoscopic wide-field microscope used with a prismatic device for viewing the container from beneath has made examination more rapid and economical. This report describes a prismatic device, which has been useful in cultivations in vitro and other general parasitological work.

The device consists of a quarter-inch plywood framework for holding the prisms and providing a suitable supporting surface for the culture containers (fig. 1, E). A wing (W) protruding from the right side of the main prism framework holds the culture tube. A slight incline (1°) in the wing restricts the tube contents (2 ml) to the area above the prism and also prevents the media from contacting the rubber cap or stopper. The wing is constructed short enough to allow the cap portion of the tube to protrude. The cap can then be used as a knob for revolving or making left and right movements of the tube. Nails at the wing tip restrict the forward or backward movements of the tube.

A length of three-quarter-inch angle iron (A.I., fig. 1, E) acts as a counterweight for stabilizing the wing. The weight provides additional surface for larger flasks and serves as a handle for positioning the device under the stereoscopic microscope.

The glass container is placed on the distal end of the prism and viewed with a stereomicroscope through the proximal end of the prism (fig. 1, C). At least two models of stereoscopic microscopes are known to be satisfactory and have produced magnifications up to 45 diameters. The limiting factor in magnification is the distance traversed through the prisms of the device and the wall of the culture container. This distance cannot be greater than the working distance of the stereoscopic microscope.

This device has made it possible to follow many characteristics in growth in vitro such as increasing size, bursal development from the fourth to the adult stage, buccal capsule changes, vitality, and survival. Thus, we can assess the growth before removing the culture from its container. After removal, obscure structures can be verified with a conventional microscope. The device can be used in other ways such as checking vial-stored specimens or centrifuge tubes for larvae left in transferring procedures.

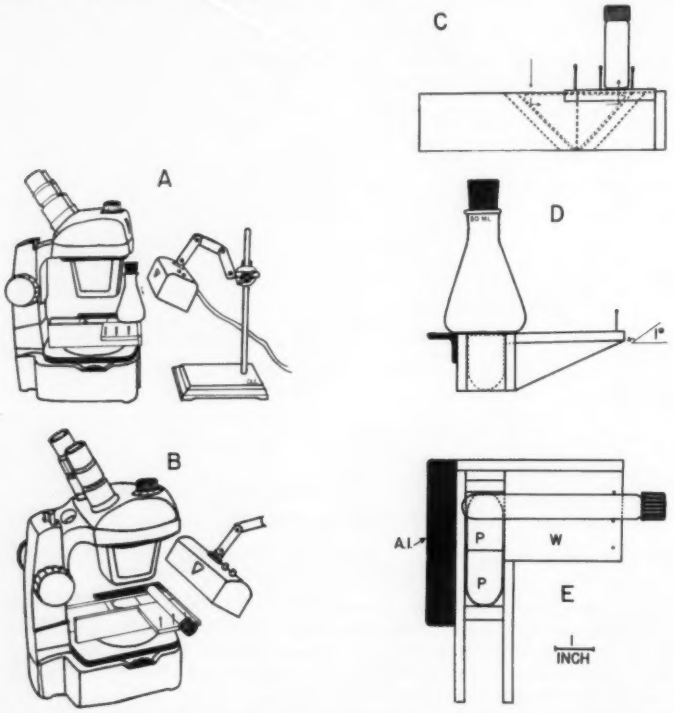

FIGURE 1. Prismatic device for viewing nematode parasites in culture in vitro. A, prismatic device under stereomicroscope showing flask in position; B, tube in position; C, position of vial over prism (side view); D, flask over prism (front view); E, framework for holding prisms (top view). A.I., three-fourths-inch angle iron; P, prism; W, wing.

Because the device rests on the stage of the stereomicroscope, it is easily removed. The stereomicroscope is then available for other purposes. The construction cost is slight. The prisms are the most expensive item, but they can sometimes be obtained economically at military surplus sources.

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THE LIFE CYCLE OF *PLAGIORCHIS GOODMANI* N. COMB. (TREMATODA: PLAGIORCHIIDAE)*

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A new xiphidiocercaria, *Cercaria goodmani,* was previously described from snails (*Lymnaea palustris*) collected from temporary ponds in the vicinity of Ann Arbor, Michigan (Najarian, 1952a). Characteristics of the cercaria placed it in Sewell's (1922) modified Polyadena group and in Brook's (1943) Conniae division of Polyadena cercariae. Natural metacercarial infections of caddis-fly larvae (*Limnephilus*) and occasional precocious metacercarial development in sporocysts of *L. palustris,* both collected from the same area, were shown to be derived from *C. goodmani* (Najarian, 1952b). The experimental encystment of *C. goodmani* in tricopterous larvae was also reported (Najarian, 1952c). This paper presents the experimental life cycle of *C. goodmani* in the laboratory, and its development into a plagiorchiid trematode, *Plagiorchis goodmani.*

MATERIALS AND METHODS

Over 4,000 *Lymnaea palustris* were collected over a 2-year period and individually isolated in small bottles of aerated tap water and observations made on cercarial emergence. No attempt was made to determine the percentage of infected non-shedding snails. Snails were maintained and reared on boiled lettuce or stained spinach. The cercariae were studied in the living state and with the usual methods of intra-vitam staining. As is true with other types of cercariae (Najarian, 1954), freshly emerged organisms were most suitable for studying alive. The temporary mount of Goodman (1951) was used in making measurements.

Field-collected caddis-fly larvae reared in a large water tank, given a lettuce diet, required 6 to 8 weeks to become adults. Cheesecloth placed over the tank trapped the adults, which were easily picked off and used for infection experiments.

Canaries, week-old chicks, white mice and rats, and various species of frogs and salamanders were tried as experimental hosts. All were refractive to infection except the canary and chick. Administration of metacercariae was by force-feeding of decapitated caddis-fly larvae and adults.

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*A part of this study was made at the Department of Zoology, University of Michigan, Ann Arbor.

Young laboratory-reared snails were infected either by de-egging worms of experimental infections, incubating the eggs at room temperature, and then placing the snails in a small volume of water with the incubated eggs, or simply by crushing adult worms followed by incubation at refrigeration temperature, after which young snails would eat the crushed worms containing the incubated eggs.

All stages in the life cycle were studied in the living state, from stained wholemount preparations and sections.

Unless otherwise indicated, all measurements given are averages in millimeters, and are based on at least 10 specimens fixed in hot 10 percent formalin. Drawings were made with the aid of camera lucida or freehand.

STAGES IN THE LIFE CYCLE

The Adult (Figs. 1–3, 9–13)

Plagiorchis goodmani (Najarian, 1952) n. comb.

Diagnosis: Body lanceolate, somewhat flattened, length 3.4; greatest width at mid-body 1.0; spines cover entire body except extreme posterior end; oral sucker subterminal, diameter 0.252; acetabulum diameter 0.226; prepharynx short; pharynx 0.120 in diameter; short esophagus bifurcating into two intestinal ceca just posterior to oral sucker; each cecum extending posteriorly along lateral margin of worm and ending blindly near posterior tip of body; ovary sub-spherical, 0.345 in diameter, just posterior to acetabulum; oviduct short, arising from posterior border of ovary; oviduct enlarging into true seminal receptacle containing many spermatoza; Laurer's canal S-shaped, entering lateral side of seminal receptacle and opening on dorsal surface of body; common vitelline duct entering oviduct just posterior to seminal receptacle; ootype and Mehlis' gland present; uterus extending posteriorly between two testes and shortly before posterior end of body, looping on itself and extending anteriorly between testes, making a wide loop at anterior border of posterior testis; uterus then extending anteriorly between anterior testis and ovary, making wide loop on itself to right of ovary, then extending to metraterum; metraterm lying lateral to acetabulum, and opening into female genital pore just to left of mid-line anterior to acetabulum; vitellaria follicular, follicles consisting of several cells each, extending from posterior border of oral sucker to posterior end of body, mostly extracecal, vitelline fields not confluent posterior as one field except dorsally posterior to posterior testis; vitel-

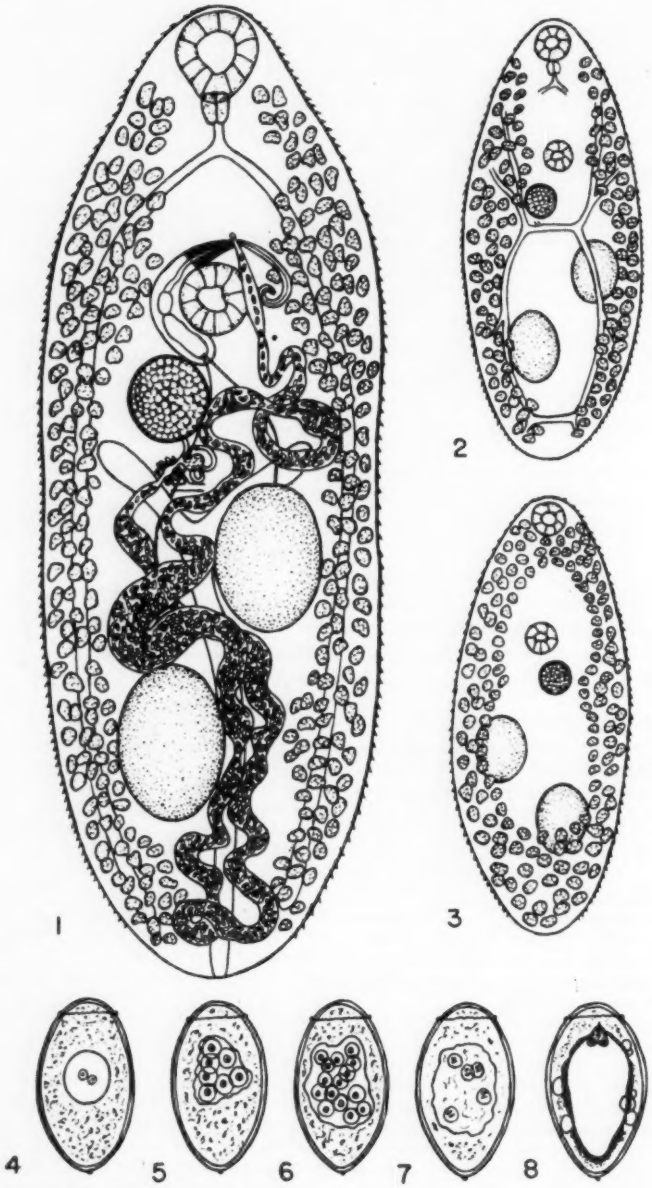

FIGURE 1. *Plagiorchis goodmani*, ventral view.
FIGURE 2. Mature worm, ventral view, showing vitellaria distribution and vitelline duct system.
FIGURE 3. Mature worm, showing vitellaria distribution from dorsal aspect.
FIGURE 4. Uncleaved egg from uterus of mature worm.
FIGURES 5–7. Eggs incubated in water for 2, 3, and 4 days respectively.
FIGURE 8. Miracidium within egg shell at 5 day's incubation.

line duct system prominent, H-shaped, with common vitelline duct just posterior to ovary; eggs (0.047 × 0.030) numerous and leaving female genital pore uncleaved; testes two, large, ovoid, in posterior half of body, oblique; posterior testis (0.704 × 0.547) slightly larger than anterior testis (0.571 × 0.518); single vas efferens leaving anterior border of each testis and entering posterior end of cirrus sac; cirrus sac long (0.532 × 0.105), elongate, extending around lateral rim of acetabulum; seminal vesicle *interna* in two unequal compartments; prostate gland cells present in anterior part of cirrus sac; cirrus long (0.456 × 0.032), protrusible, without spines or pustules; male genital pore opening just posterior and lateral to female genital pore; common genital atrium or pore absent; excretory bladder Y-shaped, with stem extending to midlevel of anterior testis; excretory pore terminal.

Hosts: Canary and chick (experimental); natural host unknown.

Location in Host: Small intestine.

Locality: Ann Arbor, Michigan (non-adult stages).

Type specimens: Hypotypes deposited in the Helminthological Collection, U. S. National Museum (No. 56065).

Egg and Miracidium (Figs. 1, 4–8, 10–14.)

The eggs are operculate and measure 0.047 long by 0.025 wide. They are ovoid and wider at the opercular end. The operculum is thickened at its base and its articulating border is denticulate. There is a small knob-like prominence at the posterior end of the egg shell. The eggs leave the uterus of the adult worms in an uncleaved condition, having a germinal area with two pronuclei surrounded by a clear area. Within the egg, most of the vitelline cells are broken down into a granular mass.

Eggs incubated in tap water developed fully matured miracidia in 4 to 5 days. Development proceeded slowly until about 48 hours after which divisions went quickly. Miracidia were never observed to hatch, and required ingestion by snails for further development into sporocysts. The miracidium is pear-shaped measuring 0.03 long by 0.018 wide. There is a prominent, eversible, glandular papilla at the anterior end; a pointed stylet is present. The two elongate penetration glands enter the apical papilla medially at its posterior end. The germinal elements are located in two areas, one anteriorly and the other posteriorly. The entire body is covered with cilia, and although three tiers of epidermal plates were observed, their number and arrangements were not determined.

Mother Sporocyst (Figs. 15, 17)

Although some egg shells were found in the feces of *Lymnaea palustris* as early as 3 hours after the snails ingested incubated worm fragments, mother sporocysts were not observed in snails before 5 days. Apparently the development of primary sporocysts takes place slowly. They were recovered from the digestive gland of the snail. They are ovoid-sacculate measuring 0.080 long by 0.055 wide. Incipient daughter sporocysts develop from distinct germinal areas at each end of the mother sporocysts. These two germinal areas are fixed by a limiting membrane, whereas the germinal elements in the cavity of the mother sporocysts are loose. Birth pores are absent and presumably daughter sporocysts escape by rupture of the wall of the mother sporocysts.

Daughter Sporocysts (Figs. 16, 18–21, 24)

Daughter sporocysts were found as early as 16 days after the snails were exposed to eggs. They are elongate, measure 0.300 long by 0.045 wide, often have swellings at one end; the wall has clumps of orange granules. Sporocysts were located extensively throughout the digestive gland of infected snails. As many as 65 developing cercarcial embryos were found in a single daughter sporocyst. A birth pore is not present, and mature cercariae presumably escape by rupture of sporocyst wall.

Cercaria

The cercaria of *Plagiorchis goodmani* has been previously described (Najarian, 1952a). It is a complete spinose xiphidiocercaria, 0.180 by 0.082, with eight pairs of large penetration glands on each side of the acetabulum, caudal pockets with spines, bicornuate excretory bladder with main collecting ducts entering the bladder subterminally, and a flame cell pattern of $2\ [(3+3+3)+(3+3+3)]=36$ flame cells. The first emergence of cercariae from experimentally infected snails was variable and occurred between 22 and 35 days. They were found abundantly in the lung cavity of the snail and escaped via the pulmonary aperture. The average infection rate (cercarial shedding) of field-collected snails was about 12 percent.

Metacercaria (Figs. 22, 23, 25, 26)

Metacercariae were found naturally in caddis fly larve (*Limnephilus indivisus*) and occasionally in sporocysts within *L. palustris*. The young cysts were oval, measuring 0.100 by 0.115, and had a thin hyaline wall. In newly encysted cercariae the penetration glands and spined

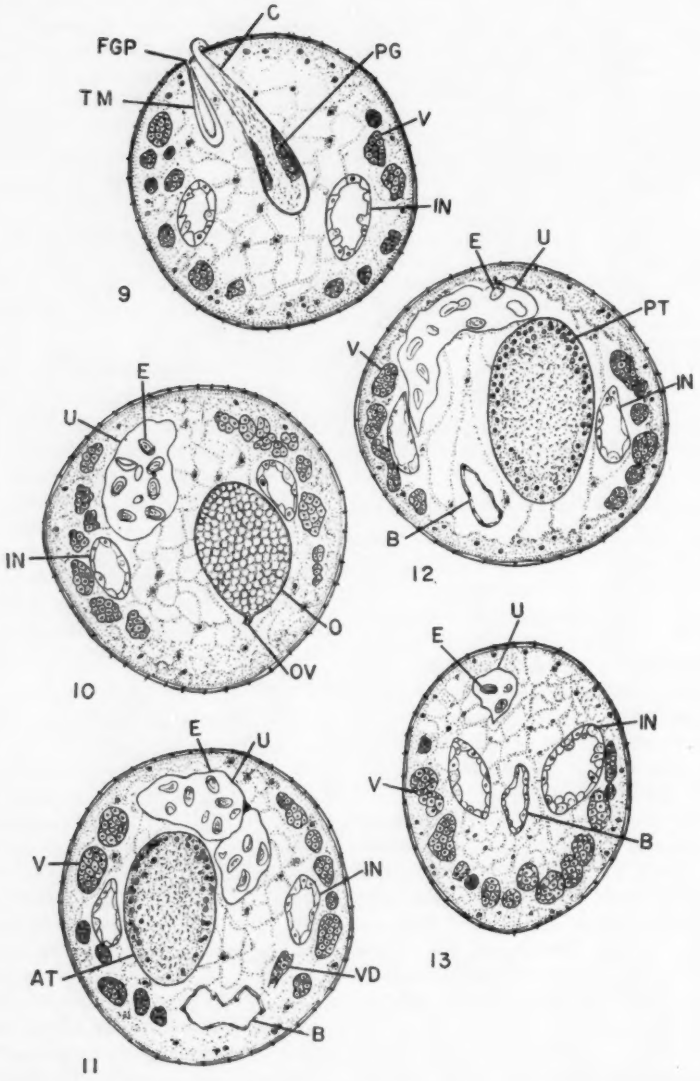

FIGURE 9. Transverse section of mature worm through region just anterior to acetabulum.

FIGURE 10. Transverse section through ovary.

FIGURE 11. Transverse section through anterior testis.

FIGURE 12. Transverse section through posterior testis.

FIGURE 13. Transverse section through posterior region of worm. Abbreviations: AT, anterior testis; B, excretory bladder, C, cirrus sac; E, egg; FGP, female genital pore; IN, intestinal cecum; O, ovary; OV, oviduct, PG, prostate gland cell; PT, posterior testis; TM, metraterm; U, uterus; V, vitallaria; VD, vitelline duct.

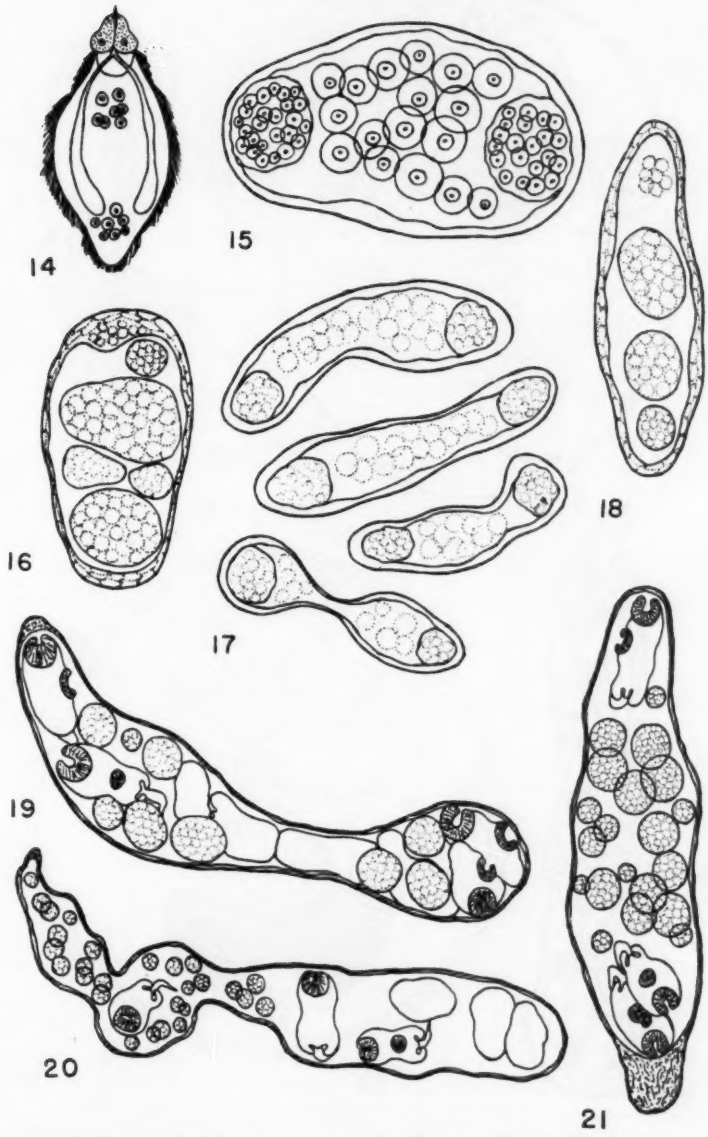

FIGURE 14. Miracidium, showing apical papilla, stylet, penetration glands, and germinal cells.
FIGURE 15. Mother sporocyst at 6 days.
FIGURE 16. Daughter sporocyst at 26 days.
FIGURE 17. Four mother sporocysts at 6 days.
FIGURE 18. Daughter sporocyst at 26 days.
FIGURES 19–21. Daughter sporocysts, showing various stages in the development of cercariae.

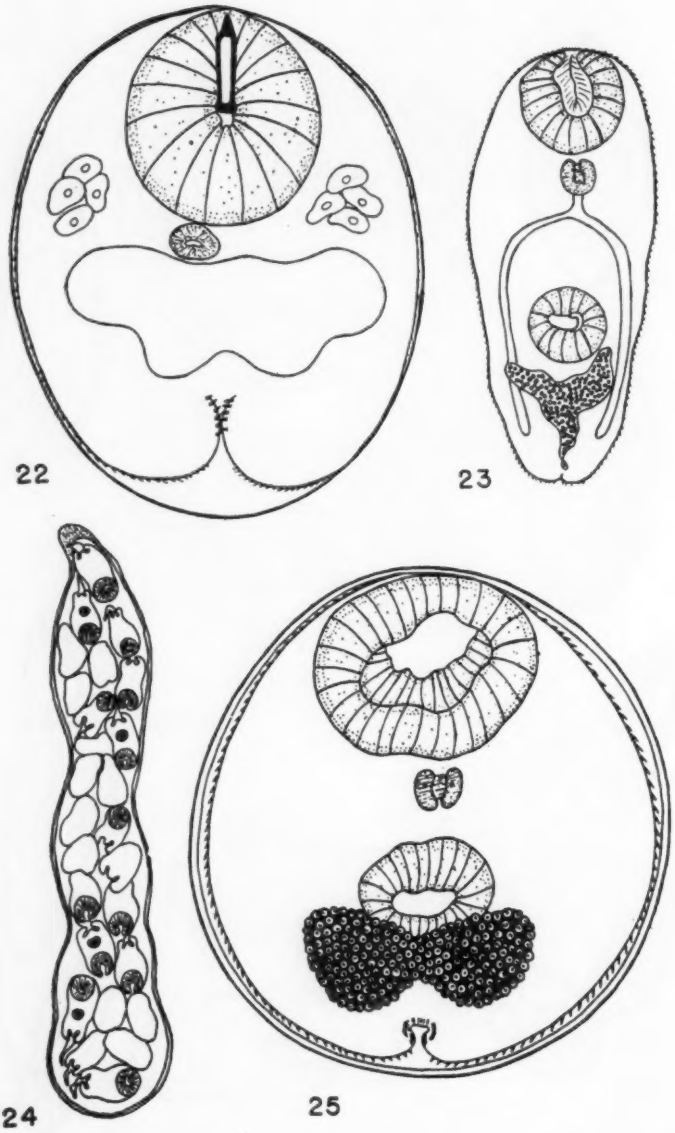

FIGURE 22. Metacercaria removed from abdomen of caddis-fly larva 9 hours after exposure to cercariae; stylet, penetration glands and caudal pockets still visible.

FIGURE 23. Metacercaria teased from cyst of naturally infected caddis-fly larva.

FIGURE 24. Daughter sporocyst with numerous cercariae.

FIGURE 25. Older metacercaria from naturally infected caddis-fly larva; heavy concretions in excretory bladder.

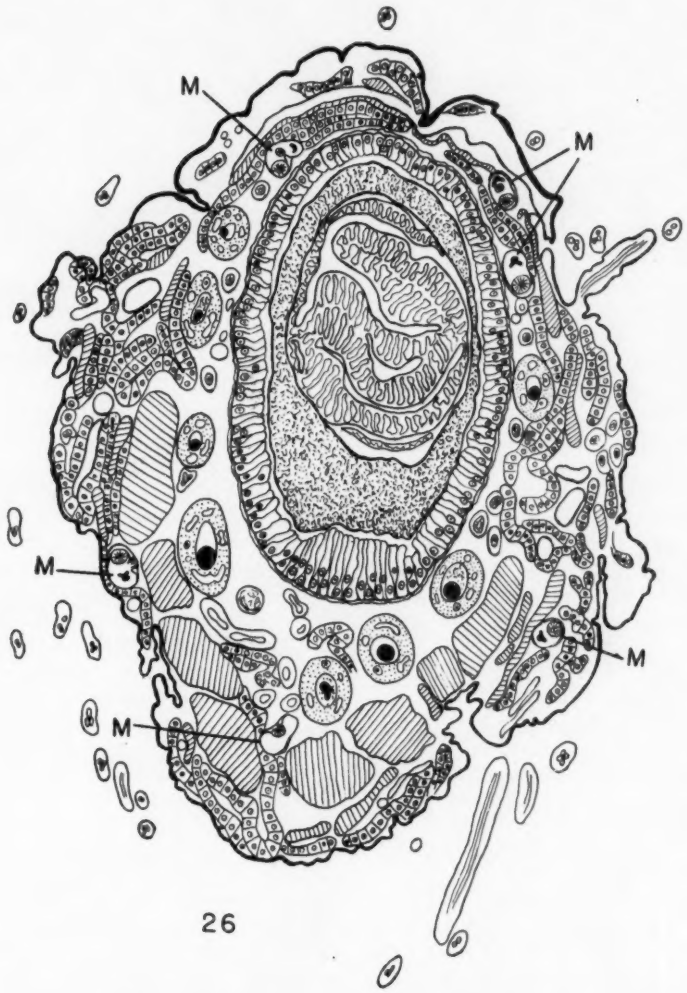

FIGURE 26. Section through abdomen of caddis-fly larva, showing location of metacercarial cysts. Abbreviation: M, metacercaria.

caudal pockets were still visible, the stylet still attached, and the excretory bladder relatively free of concretions. Older cysts were larger and more spherical (0.130 to 0.190 in diameter), the stylet became loose and disappeared, the cyst wall was thicker and surrounded by pronounced reaction from host tissue, the penetration glands disappeared, and the excretory bladder became heavy with concretions. Larvae exposed for 15 minutes to cercaraie and examined immediately thereafter showed as many as 167 freshly encysted metacercaraie located mostly in the fatty tissue of the abdomen. Observations on cercariae prior to encystment were previously reported (Najarian, 1952c). Caddis-fly larvae exposed to cercariae and reared in the laboratory to the adult stage demonstrated that metacercariae remain viable and infective for at least two

months.

Development of the Adult

Canaries proved to be excellent experimental hosts for the development of *P. goodmani*, and permitted observations on worms until they reached maturity.

At 48 hours worms recovered from the small intestine measured 0.672 long by 0.266 wide. The pharynx was 0.055, the oral sucker 0.106, and the acetabulum 0.086 in diameter, respectively. The cirrus sac, ovary, and testes were distinct. The ovary measured 0.043 in diameter and the two testes were of about equal size (0.115 long by 0.082 wide), and were almost side by side.

At 6 days the worms measured 1.62 long by 0.478 wide. The oral sucker, acetabulum, and pharynx measured 0.186, 0.159, and 0.92 in diameter, respectively. The ovary measured 0.133 in diameter, and the two testes were more oblique in relation to each other. The posterior testis (0.279 by 0.199) was larger than the anterior testis (0.226 by 0.172). Vitelline follicles were present but not extensive and the uterus was not completely formed.

At 11 days the worms were mature, measuring 2.1 by 0.65. The seminal vesicle, viable sperm, Laurer's canal, complete vitelline fields, fully developed uterus and metraterm, and numerous eggs were all present. Additional growth of the worms and organs took place beyond 11 days, but this interval in the intestine of the canary was sufficient for the development of worms which produced eggs.

The average recovery of adult worms, based on feeding experiments with eight canaries, was 22 percent. Eggs were recovered from canary feces as early as 14 days. Burdens of over 200 mature worms were responsible for the quick death of three birds. On autopsy, the small intestine was very much involved and inflamed to the point that worm locations could be picked out before exposing the intestinal lumen. In birds with considerably fewer worms, the infection was lost in 4 to 6 weeks.

An examination of a few specimens of the following vertebrates from the woods-pool area showed no infection with *P. goodmani: Peromyscus sp.*, muskrat (*Ondatra zibethicus*), eastern kingbird, robin, blue-gray gnateatcher, eastern towee, red-winged blackbird, purple grackle, song sparrow, indigo bunting, red-eyed vireo, catbird, crow, tufted titmouse, wood peewee, blackcap chickadee, goldfinch, solitary sandpiper, and bluewinged teal.

DISCUSSION

Excepting *Plagiorchis megalorchis* Rees, 1952, *P. goodmani* differs from all hitherto decribed species in the genus by having seperate genital pores for the male and female reproductive systems. The adults of *P. goodmani* and *P. megalorchis* may be distinguished on the basis of body and organ size, extent of body spination, position of testes, ovary, cirrus sac, genital and excretory pores, and presence of seminal receptacle.

P. goodmani may be distinguished from most other species in the genus by its definite seminal receptacle. The only other described species with a seminal receptacle is *P. noblei* Park, 1936, which differs from *P. goodmani* in the distribution of vitellaria and body spination, size of testes, and presence of genital atrium. In systematic accounts *P. muris* (Tanabe, 1922) Yamaguti, 1933 is usually described as lacking a seminal receptacle, but in the translation of Tanabe's original description Dolfuss (1925) stated that in *P. muris* there is a small diverticulum representing the seminal receptable. Yamaguti (1958) stated that the seminal receptacle may be either present or absent in the genus *Plagiorchis*. The present study not only agrees that this character should be included in the generic diagnosis, but also suggests that the presence of one or two genital pores be considered as characters of the genus. Size of testes, nature of the uterus, distribution of vitellaria, length of cirrus sac, and position of ovary further distinguish *P. goodmani* from *P. muris*.

P. goodmani differs from closely related species, *P. microcanthos* Macy, 1931, *P. jaenschi* Johnston and Angel, 1951, *P. vespertilionis* (Müller, 1784) Braun, 1900, *P. vespertilionis parorchis* Macy, 1960, *P. proximus*, Barker 1915, in the number of genital pores, presence of seminal receptacle, size and distribution of body spines, distribution of vitellaria, size and position of testes and ovary, and position and extent of uterus.

Although the cercaria of *Opisthioglyphe locelus* Kossack, 1910, shows considerable resemblance to the cercaria of *P. goodmani*, the experimental life cycle of that cercaria (Macy and Moore, 1958) demonstrated its develop-

ment into a trematode of the Subfamily Omphalometrinae Looss, 1899, having distinctly different adult characters. The cercaria of *P. goodmani* differs from the cercariae of *O. locellus, P. megalorchis, P. muris, P. microcanthos, P. vespertilionis,* and *P. jaenschi* in body size, presence of large cells and caudal excretory duct in the tail, distribution of spines on body and tail, position of attachment of main collecting ducts to the excretory bladder, presence of body bristles, shape of the stylet, posterior extent of the intestinal ceca, presence of caudal pockets with spines, and size of cystogenous gland cells. In the original description, Cort (1914) did not describe nor figure caudal pockets with spines in the tail region of *Cercaria polyadena.* However, in a later paper (Cort, 1919) on the excretory system of the same cercaria, caudal pockets were described as being present. Despite this discrepancy, the cercaria of *P. goodmani* can be distinguished from the cercaria of *P. proximus* = *Cercaria polyadena* on the basis of body and tail size, distribution of body spines, shape of the stylet, and number of penetration glands.

The life cycle of *P. arcuatus* Strom, 1924, has recently been described (Paskalskaya, 1954, 1955), but since the literature is not available, comparison of larval forms of this species with *P. goodmani* is not possible.

The genus *Plagiorchis* Lühe, 1899, occurs in a variety of birds and mammals and to a much lesser extent in reptilian, amphibian, and piscine hosts. Systematic accounts of the genus and the family Plagiorchiidae have been given by Pratt (1902), Baer (1924), Massino(1927), Schulz and Skworzow (1931), Mehra (1931), McMullen (1937), Olsen (1937), and Yamaguti (1958). The division of the genus into two subgenera, viz., *Plagiorchis* and *Multiglandularis,* on the basis of the distribution of vitellaria anterior to the acetabulum does not seem desirable. This opinion is based primarily on the fact that vitelline follicles of trematodes, especially plagiorchids, are not distributed similarly when viewed from the dorsal and ventral aspects. Furthermore, most descriptions of vitellaria fail to mention the referential side of the trematode Olsen (1937) and Rees (1952) accepted the *Plagiorchis-Multiglandularis* division of the genus, but Yamaguti (1958) did not recognize the subgenera.

SUMMARY

The life cycle of *Plagiorchis goodmani* was established experimentally in the laboratory and all stages described. The eggs leave the uterus of the adult worm in an uncleaved condition. When incubated in tap water at room temperature, miracidia developed in 4 to 5 days. Eggs having miracidia must be eaten by *Lymnaea palustris* for the transformation into sporocysts. Mother and daughter sporocysts developed in the digestive gland of the snail, the latter giving rise to cercariae which emerged from snails 22 to 35 days after eggs were eaten. Cercariae penetrated and encysted in caddis fly larvae (*Limnephilus indivisus*) and remained viable and infective through metamorphosis of the adult insect. Metacercariae fed to canaries and chicks developed into adults in the small intestine in 11 days.

Although the natural vertebrate host is not known, naturally infected snails and caddis-fly larve were found. *Cercaria goodmani* Najarian, 1952, is the larval stage.

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RESEARCH NOTE

ON THE LIFE CYCLE OF *CLEIDODISCUS PRICEI* MUELLER, 1936.

Initial skin scrapings from *Ictalurus catus* Linnaeus, 1758, taken from the Sacramento River drainage, yielded several very small immature dactylogyrids. Although these specimens possessed fourteen characteristic hooks on the rear end of the body, they lacked a differentiated haptor, anchors, vitellaria, and copulatory complex. Suspicion that these were developmental forms of the prevalent adult species (*Cleidodiscus pricei*) was confirmed upon discovery of additional specimens that furnished a developmental series from the original "larval" form on the skin to the adult that infests the gills. The haptoral hooks on the youngest specimen (s) did not possess a base. The second specimen in the series, in addition to possession of the fourteen haptoral hooks, exhibited a differentiated point and a distal portion of each the ventral anchor shafts. The third member of the series was similar to that of the second except that the point and shaft (but not the base) of each ventral anchor were well developed, and in addition each point and a short distal portion of each dorsal anchor shaft were evident. The fourth specimen in the series was obtained from the gills and possessed the normal haptoral armament of the adult although the hook bases were short and subspherical. Further the copulatory complex was absent as in all previous developmental forms in this series. The haptoral armament of the fifth stage of development was similar to that of the fourth. The cirrus, however, was present for the first time and, although fully developed, only the distal half of the accessory piece was differentiated. The adult of *C. pricei* differs little from the fifth member of the developmental series except that the hook bases are elongate-ovate, the vitellaria are fully developed, the accessory piece spans the arc of the cirrus and articulates with the base of the latter structure, and the specimens are of a larger size.

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THE IDENTITY OF *ECHINOPARYPHIUM FLEXUM* (LINTON, 1892) DIETZ, 1910 (TREMATODA: ECHINOSTOMATIDAE)

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In a study on growth, development and survival of echinostomes, Senger (1954) considered *Echinoparyphium flexum* (Linton, 1892) Dietz, 1910, synonymous with *Echinoparyphium recurvatum* (von Linstrow, 1873) Lühe, 1909, but failed to give reasons for this view. The purpose of the present note is to point out the evidence which justifies the consideration of these two flukes as distinct species, and to emphasize the proper concept of the seminal receptacle as applied to trematode anatomy.

E. recurvatum collected from the European duck, *Fuligula marila,* was described by von Linstow (1873) as *Distomum recurvatum.* Lühe (1909) placed the species in the genus *Echinoparyphium* Dietz, 1909. The life cycle has been reported by Mathias (1927), Harper (1929), Azim (1930), and Rasin (1933), the latter being the most complete account. This species is found in a variety of birds and occasionally in mammals, and is cosmopolitan in distribution.

E. flexum was described as *Distomum flexum* by Linton (1892) from the intestine of the black scoter, *Oedemia americana,* collected at Yellowstone National Park. The life cycle has been given by McCoy (1928) and Najarian (1954). This species has also been reported from the blue-winged teal, *Anas discors,* and its distribution is apparently limited to North America.

The most noticeable difference in the adults of the two species is that *E. flexum* has a seminal receptacle (Najarian, 1954) and *E. recurvatum* lacks one (Rasin, 1933). Present in the first part of the uterus of both species, however, is a "receptaculum seminis uterinum," which is a part of the uterus so filled with large masses of sperm that its diameter is stretched 4 to 5 times. McCoy (1928) described it in *E. flexum* and erroneously called the structure a seminal receptacle. Other authors have also mistaken the "receptaculum seminis uterinum" for a seminal receptacle. It is emphasized that the latter term should be applied only to that structure which is an enlargement of the oviduct or Laurer's canal, and that the "receptaculum seminis uterinum" is simply a modified portion of the uterus.

Of the species described in the genus *Echinparyphium, E. elegans* (Looss, 1899) Dietz, 1909 and *E. flexum* are the only ones which have a true seminal receptacle and a "receptaculum seminus uterinum." Although Looss (1899) did not describe the seminal receptacle in *E. elegans,* it is clearly indicated in his figure. *Hypoderaeum conoidium* (Block, 1782) Dietz, 1909, has a large seminal receptacle which in gross appearance looks like the "receptaculum seminis uterinum" of other echinostomes, but Rees (1932) has demonstrated that it is a true seminal receptacle, being an extension of the oviduct and entering directly into the ootype. The lack of a true seminal receptacle and the presence of a "receptaculum seminis uterinum" is characteristic of the genus *Echinostoma* Rudolphi, 1809. However, Nasir (1960) has recently described *Echinostoma nudicadatum* in which a seminal receptacle is present, so that this character may be variable in *Echinostoma* species. Thus, there are three types of situations in echinostomes with respect to the seminal receptacle: (1) Those species in which there is present only a "receptaculum seminis uterinum" (most *Echinostoma* and various echinostome genera); species in which both a true seminal receptacle and "receptaculum seminis uterinum" are present (*E. elegans, E. flexum*); and (3) species in which a large seminal receptacle is present and a "receptaculum seminis uterinum" is absent (*Hypoderaeum conoidium*).

The miracidia of *E. flexum* and *E. recurvatum* differ in several respects. In addition to being considerably smaller than *E. recurvatum,* the miracidium of *E. flexum* has a single pair of eye spots (Najarian, 1954), whereas *E.*

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recurvatum has two pairs of eye spots (Rasin, 1933). Although the miracidium of both species has four tiers (18 plates) of epidermal plates, 6–6–4–2, in *E. flexum* there is a prominent papilla between the first and second tiers on each lateral margin (Najarian, 1954). Rasin (1933) has reported these structures lacking from *E. recurvatum;* they are also absent in the case of *E. revolutum,* (Johnson, 1920; Beaver, 1937). A further point in the distinction of the miricidia of *E. flexum* and *E. recurvatum* is that in the former the third tier of epidermal plates do not border on the second tier, so that there is a naked area on which no cilia are present. This region is quite noticeable in living miracidia but is more apparent in silver nitrate preparations. Such a condition is lacking in the miracidia of *E. recurvatum, E. revolutum,* and *Euparyphium ilocanum.*

The cercariae of *E. flexum* and *E. recurvatum* may be distinguished by the number of gland openings on the anterior tip of the oral sucker (*E. flexum* has four pairs, *E. recurvatum* has three pairs). Additional differences are based on the distribution of body spines and the condition of the excretory tube in the tail (Najarian, 1954).

Based on the characters of the adult, miracidial, and cercarial stages, it is evident that *E. flexum* and *E. recurvatum* are distinct echinostome species. Furthermore, it is obvious that conclusions with respect to synonymy of species should be accompanied by factual information, rather than by opinion alone. Attention is also directed to the proper usage of the term seminal receptacle in echinostome morphology.

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STUDIES ON BOVINE GASTROINTESTINAL PARASITES. XXIV. RESULTS OF SOME PHENOTHIAZINE, COPPER SULPHATE AND NICOTINE SULPHATE EXPERIMENTS

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The following experiments were suggested because of an increasing number of complaints on the failure of anthelmintics to control parasites.

MATERIALS AND METHODS

The animals used in the mixed infections were four naturally infected 9- to 12-month old heifers in the Louisiana State University dairy herd. The species of parasites with which they were infected were determined by egg examinations according to Krug and Mayhew (1949) and; they consisted of *Cooperia punctata*, nodular-stomach worm groups, and hookworms. A very small number of *Nematodirus* sp. eggs were regularly recovered from Nos. 347, F61, and F70.

The three animals used in the controlled infections were males confined in the specially constructed cages in the Veterinary Science Department and were cared for as described by Mayhew et al (1960b). They were artificially infected with pure cultures of *Cooperia punctata* and *Oesophagostomum radiatum*, as described under each experiment.

The phenothiazine used in the low-level feeding experiments was of the NF grade and fed at the 1.5 g per day level. A widely used commercial drench preparation of phenothiazine was used in the therapeutic experiments. The copper sulphate and nicotine sulphate were in standard therapeutic doses.

RESULTS

The same experiments were carried out on Nos. F61, F70, 347, and 614, and the results on the first three are shown in figures 1, 2, and 3. These were 9- to 12-months-old heifers belonging to the L.S.U. Dairy Department. One and one half g of N.F. grade phenothiazine per day was fed by mixing the drug with the grain ration as indicated in the graphs. The large stomach-nodular worm eggs disappeared as expected (Mayhew 1952 a, b). No changes were noted in the *Cooperia punctata*, hookworm, or *Nematodirus* sp. egg counts (the latter not shown).

A commercial drench preparation of phenothiazine was administered at the rate of 10 g of the drug per 100 lbs body weight on the dates indicated in the graphs. The continuous production of eggs of *Cooperia punctata* and hookworms indicates that the drug at this dosage level had no effect on these species.

Six ounces of 1 percent solution of copper sulphate was then administered to each animal on the dates indicated. Six ounces of a 1 percent solution of copper sulphate and nicotine sulphate was then given each animal. It will be noted that neither of these anthelmintics was effective as measured by numbers of eggs of *Cooperia punctata* and hookworms.

Calf No. 614. The same series of experiments were carried out on this animal as on the above, but the details are not shown in the figures. The eggs recovered belonged to the same genera of parasites as those found in the other three animals. Twenty-four counts made between October 13 and December 2 showed the Cooperia eggs varying between 2 and 60 per g. The hookworm eggs varied from 0 to 1 per g. The stomach-nodular group of eggs varied from 1 to 5.75 eggs per g between October 13 and 30. Following October 30 the latter decreased rapidly and disappeared entirely due to the feeding of 1.5 g of phenothiazine begun on October 21. Ten grams of phenothiazine per 100 lbs body weight was administered on November 3, 6 oz of 1 percent $CuSO_4$ on November 12, and 6 oz 1 percent $CuSO_4$ to which 1 percent nicotine sulphate had been added on November 19. As in the case of the above three calves, there was no effect on the egg counts of *Cooperia punctata* or of the hookworm.

Controlled Infections

No. 277. This animal was kept under controlled conditions of infection and had been inoculated with *Cooperia punctata*. Ten grams of phenothiazine per 100 lbs body weight was administered July 2, 1955 (fig. 4). Eleven days later a sharp decrease in egg counts occurred lasting from July 14 to 29. We believe this was an attempt to develop immunity, since it

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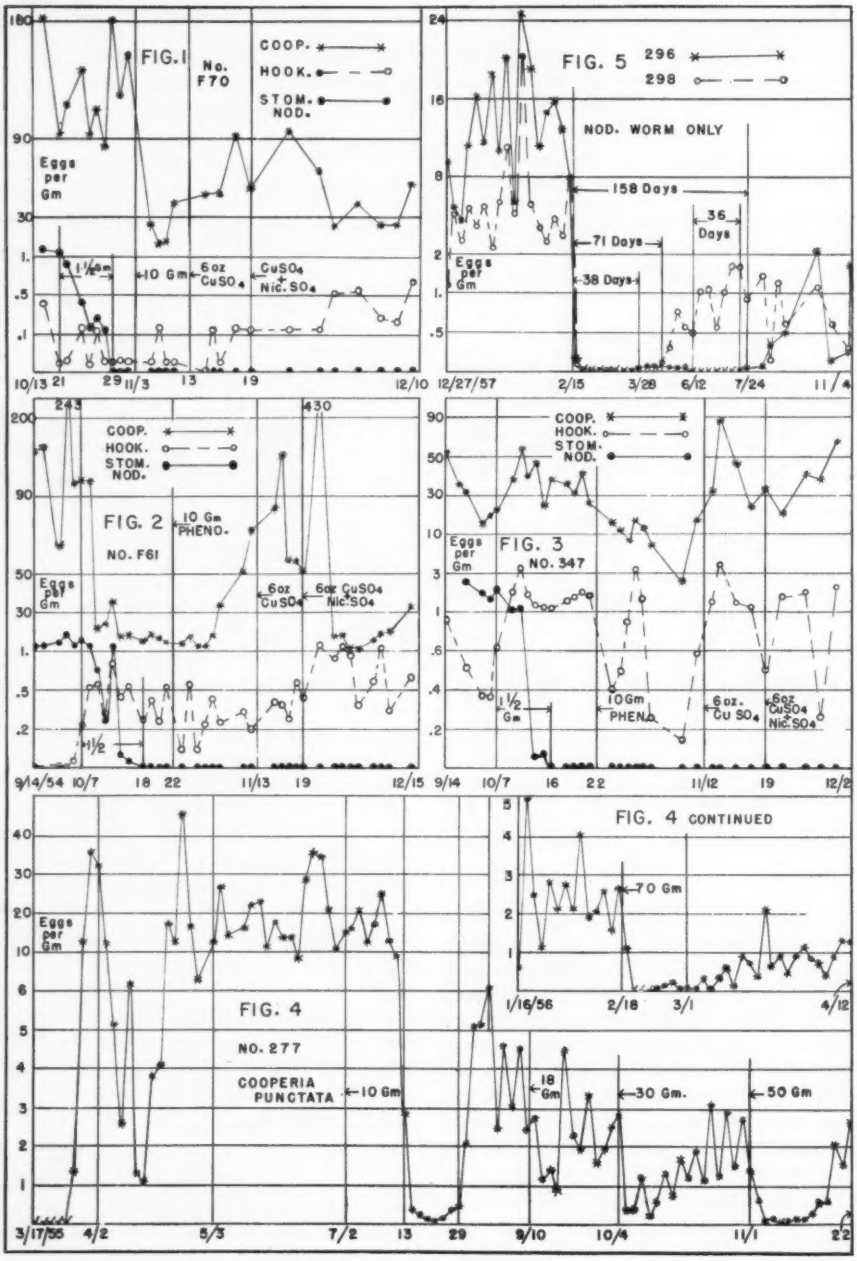
FIG. 1
No. F70
COOP.
HOOK.
STOM. NOD.
Eggs per Gm
1½ Gm
10 Gm
6oz CuSO4
CuSO4 + Nic. SO4
10/13 21 29 11/3 13 19 12/10
FIG. 5
296
298
NOD. WORM ONLY
158 Days
71 Days
38 Days
36 Days
Eggs per Gm
12/27/57 2/15 3/28 6/12 7/24 11/4
FIG. 2
NO. F61
COOP.
HOOK.
STOM. NOD.
10 Gm PHENO.
6oz CuSO4
6oz CuSO4 Nic. SO4
Eggs per Gm
243
430
1½
9/14/54 10/7 18 22 11/13 19 12/15
FIG. 3
NO. 347
COOP.
HOOK.
STOM. NOD.
Eggs per Gm
1½ Gm
10 Gm PHENO.
6oz. Cu SO4
6oz CuSO4 Nic. SO4
9/14 10/7 16 22 11/12 19 12/2
FIG. 4
NO. 277
COOPERIA PUNCTATA
Eggs per Gm
10 Gm
18 Gm
30 Gm.
50 Gm
3/17/55 4/2 5/3 7/2 13 29 9/10 10/4 11/1 22
FIG. 4 CONTINUED
70 Gm
1/16/56 2/18 3/1 4/12

occurred too late to be due to the action of the drug. We have observed many animals react in this way to *Cooperia* and other species of parasites.

In an attempt to determine if larger amounts of the drug would be effective in removing the parasites, 18, 30, 50, and 70 g of phenothiazine per 100 lbs body weight were then administered as indicated (fig. 4). It will be noted that there was more or less temporary reduction in egg counts in each instance followed by return to the former level.

Oesophagostomum radiatum

Nos. 296 and 298. On November 1, 1957, these animals were inoculated with nodular worm larvae and, on schedule, nodular worm eggs appeared (fig. 5). On February 15, 1958, phenothiazine was administered at the rate of 10 g per 100 lbs body weight to both animals. There was a sharp drop to nearly 0 the next day and on the 2d day no eggs were recovered from either animal. Number 296 remained negative for 39 days. A few eggs were recovered (.03 to .08 E.P.G.) on six examinations between March 28 and June 5. Eleven negative examinations followed between June 12 and July 18. Eleven examinations made between July 24 (158 days after treatment) and November 4 yielded eggs varying between 0.04 and 2.12 E.P.G. A comparison of a series of 13 counts representing the range of E.P.G. preceding and following treatment shows a return to 4 percent of the original counts. No. 298 remained negative for at least 39 days and possibly as long as 71 days after treatment. No examinations were made between the 39th and 71st days. Between April 30 and November 4, thirty egg counts varied between .30 and 1.88 E.P.G. This is a return of 16.9 percent when 13 counts representing the range preceding and following treatment are compared.

That reinfection did not occur in these animals from the cages is indicated by the fact that the calves confined in the adjoining cages were not infected with nodular worms during the entire period of observation. This fact is in addition to the careful methods of care already referred to previously (Mayhew et al, 1960b).

DISCUSSION

The above results suggest that the apparent inefficiency of phenothiazine, copper sulphate, and nicotine sulphate in controlling parasite infections under field conditions is probably due to the lack of lethal action on the above mentioned species. Recent reports of finding immature stages of several species (Bailey and Herlich 1953, Herlich 1956) following treatment may be an explanation. These immature stages may then complete their development and proceed to produce eggs which result in severe infections in susceptible animals.

The fact that phenothiazine is not 100 percent effective against some species and variable in the case of others has been shown by various workers in critical tests (Mayhew 1960a, table 2). The adult worms that remain after treatment then could build up the infection and losses again occur.

SUMMARY

Four naturally infected animals were treated with 10 g of phenothiazine per 100 lbs body weight, followed by copper sulphate, followed by copper sulphate with nicotine sulphate added. The parasites present as determined by egg examinations were *Cooperia punctata, Bunostomum phlebotomum, Nematodirus spp.,* and *Oesophagostomum radiatum.* The eggs of *O. radiatum* were eliminated by the daily feeding of 1.5 g of phenothiazine. The eggs of the other

FIGURES 1, 2, and 3. Effects of phenothiazine, copper sulphate, and copper sulphate plus nicotine sulphate on egg counts of mixed infections.

FIGURE 4. Effects of various amounts of phenothiazine on egg counts in a pure infection of *Cooperia punctata.*

FIGURE 5. Effects of 10 g phenothiazine on egg counts of a pure infection of the nodular worm, *Oesophagostomum radiatum.*

(Abbreviations: $CuSO_4$, copper sulphate; Nic SO_4, nicotine sulphate; pheno, phenothiazine; gm, grams; Coop., *Cooperia punctata;* hook, hookworm, (*Bunostomum phebotomum*); Stom. Nod., stomach worm (*Haemonchus contortus*) nodular worm (*Oesophogostom radiatum* egg group.)

species were not affected either as to appearance or numbers by low-level feeding or the therapeutic treatment.

One animal experimentally infected with *Cooperia punctata* was treated with phenothiazine at dose levels of 10, 18, 30, 50, and 70 g per 100 lbs body weight. Reductions in the egg count levels occurred following each treatment (except the 10 g) but in each instance the numbers gradually increased to the former range of counts.

Two animals with pure infection of *Oesophagostomum radiatum* were treated with 10 g of phenothiazine per 100 lbs body weight. The egg count became zero the second day following treatment and remained so for 38 and 71 days, respectively. One animal then produced eggs at 4 percent and the other at 16.9 percent of the former level.

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RESEARCH NOTE

A BAT TREMATODE, *PROSTHODENDRIUM NAVICULUM* MACY, 1936, NATURALLY OCCURRING IN RACCOONS.

Two raccoons, *Procyon lotor lotor* (Linnaeus), taken in central Ohio in 1957 by Dr. Donal Myer and Mr. Robert Sagar were positive for 647 specimens of *Prosthodendrium naviculum* Macy, 1936, a trematode parasite of bats. The worms were removed from the small intestines of the two host animals. I have found this fluke to be common in bats and have obtained it from three species in Ohio and Kentucky.

Most of the worms were mature but not bulging as heavily with dark brown eggs near the metraterm as were mature specimens from bats. The egg size of these specimens agrees with the Tennessee material of Byrd and Macy (1942, Tenn. Acad. Sci. **17**: 149–156). Although the life history of *P. naviculum* is not known it undoubtedly involves an aquatic insect as the second intermediate host. It is conceivable that a raccoon could pick up metacercariae from immature aquatic insects ingested along with crayfish and fish.

Sawyer (1958, J. Parasit. **44**: 63) reported unidentified bat trematodes in poor condition from a raccoon in Georgia, but doubted whether this was a natural infestation since bats had been fed to the animal during a rabies study. I have found no other reports of bat flukes occurring in raccoons.—Russell R. Williams, *Department of Zoology and Entomology, Ohio State University, Columbus 10, Ohio.*

EXPERIMENTAL TRICHURIASIS. I. ANTAGONISM BETWEEN *TRICHURIS MURIS* AND *ASPICULURIS TETRAPTERA* IN THE ALBINO MOUSE

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It has been suggested by Shikhobalova (1937) that *Trichuris muris*, the mouse whipworm, can be used in testing substances for potential activity against *Trichuris trichiura* in man. A prerequisite of any efficient anthelmintic screen is the ability to maintain the infection at a predictable level in a high percentage of the animals employed. Using a strain of *T. muris* which had been isolated from wild grey mice, Shikhobalova was able to maintain an infection rate of 74 percent in albino mice. Unfortunately, no results of chemotherapeutic investigations appear to be available. In attempts to maintain *T. muris* in albino mice for screening purposes, our observations have produced some interesting information concerning a factor which may influence the relationships of this parasite and its host.

A serious difficulty encountered in the early experimental infections with *T. muris* was the large proportion of albino mice which appeared to be resistant to the infection. For example, during the period from July 25, 1956, to October 31, 1956, of 352 mice coming to autopsy only 125 (35.5 percent) were parasitized. Variation of infection routines and experimentation with the regulation of food and water supplies did not improve this infection rate.

The mice had been obtained from a single breeder and many of them harbored naturally acquired infections of the common mouse oxyurids *Aspiculuris tetraptera* and *Syphacea obvelata*. It was noticed that large numbers of these oxyurids were frequently present in mice which had proved refractory to *T. muris* infection, and that only rarely did large burdens of *T. muris* and oxyurids occur in the same host. As all three species inhabit the hindgut, this observation suggested that some form of interspecific antagonism might be operating to the disadvantage of *T. muris*. In order to explore this suggestion and to obtain information which might help to elucidate the mechanisms involved in such antagonism, the following experiments were performed.

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MATERIAL AND METHODS

Preparation of Mice

The albino mice used in all experiments were 3 to 4 weeks old weighing 18 to 20 g. Only one strain of mice (Clarke) was employed. In some groups of animals naturally acquired oxyurid infections were removed by the administration of piperazine phosphate by gavage at a suitable dose level. All dose levels are expressed as piperazine base.

Trichuris muris

The strain of *T. muris*, E.S., (kindly provided by Dr. C. Rayski, Department of Zoology, University of Edinburgh) was isolated from house mice, *Mus musculus*, trapped in the Zoological Park, Edinburgh.

Embryonation of eggs. Gravid females were collected at autopsy and macerated by gentle grinding in a mortar. The material was suspended in tapwater and incubated in covered 4 in-petri dishes at 27 C. Cultures were examined after approximately 35 days. The presence of well developed larvae in about 25 percent of the eggs at this time was regarded as an indication of satisfactory infectivity. Most of the macerated body tissues disintegrated during the long incubation period. Gentle scraping with a spatula was sufficient to free any eggs which adhered to the bottom of the dish and vigorous agitation produced a homogeneous suspension.

Infection of mice. Embryonated eggs in three 0.1 ml aliquots were counted under low magnification and averaged. The desired concentration was obtained by adjustment of the total volume of suspension by gavage.

Autopsy procedure. At autopsy the abdomen was opened and the cecum, colon, and rectum removed, slit open, and examined under water in a 4 in-petri dish. The adult *T. muris* were counted as they were removed individually. The anterior end of each worm was freed from the mucosa by a gentle pull on the posterior end with a pair of forceps.

Aspiculuris tetraptera

Gravid female worms were obtained from naturally infected mice. Eggs were collected, counted and administered by the methods described

for *T. muris*. The incubation period to the infective stage for *A. tetraptera* eggs was 5 to 7 days at 27 C. The autopsy procedure was similar to that for *T. muris* except that the worms were counted as they were recovered by Pasteur pipette.

Statistical Analysis of Results. The mouse infection rates were contrasted, as they seemed particularly relevant to the problem of obtaining infected animals for screening purposes. A more critical estimate of the success of infection was based on contrasts of worm burdens. As the individual worm burdens within each group were not distributed normally, it was considered unfair to base calculations of significance on the average worm burden. For this reason, contrasts were made between the numbers of animals in each group which attained an arbitrarily selected level of infection. This level was fixed at 10 specimens of *A. tetraptera* and 20 specimens of *T. muris* in each animal. Levels of significance for all contrasts were obtained from 4-fold contingency tables (Mainland, Herrera and Sutcliffe, 1956).

RESULTS

Experiment 1

As a preliminary experiment 20 mice were treated with a single dose of piperazine phosphate at a dose level of 886 mg per kg (base). Seven days later these mice and 20 untreated mice of the same age, which acted as controls, each received a dose of 250 embryonated *T. muris* eggs. All surviving mice were autopsied 48 days after infection, and the adult *T. muris* were counted. The oxyurids were not counted, but the worm burden was estimated and designated 0, +, ++, or +++. The results are shown in table I.

TABLE I. *Effect of removal of oxyurid infection from mice on subsequent infection of* Trichuris muris.

	T. muris							*A. tetraptera*
Piperazine treatment	*T. muris* infection	Number of embryonated eggs/mouse	Survivors	Mouse infection rate	Average infection (range)	Mice harboring > 20 worms	Significance level of contrasts between groups: Mouse infection rate	Significance level of contrasts between groups: Mice harboring > 20 worms	Mouse infection rate
28.3.57	4.4.57	250	18/20	14/18	46.7 (0–138)	11/18			5/18
—	4.4.57	250	16/20	4/16	9.1 (0–82)	2/16	< 1.0%	< 1.0%	13/16

The results gave support to the suggestion that the presence of oxyurids in the mice lowered susceptibility to infection with *T. muris*. The conditions of the experiment did not furnish information concerning the extent of the oxyurid infection in the control group at the time of *T. muris* infection. It was, therefore, necessary to obtain confirmatory evidence in experiments where numbers of eggs given and the time of the oxyurid infection were more strictly controlled.

Experiment 2

Two groups of 10 mice were treated with piperazine phosphate at a dose level of 443 mg per kg (base). The treatment was repeated 1 week later. On the day after the second dose all the mice in one group received 350 embryonated *A. tetraptera* eggs. One week later the mice in both groups were given 100 embryonated *T. muris* eggs. The *T. muris* burdens in all survivors were counted at autopsy 49 days later.

Experiment 3

The structure of this experiment was similar to that described for experiment 2, but the piperazine treatment was given as phosphate in a single dose of 886 mg per kg (base), and followed on the next day by an infection of 250 embryonated *A. tetraptera* eggs to 10 of the mice. All survivors were autopsied 48 days after receiving the infective dose of *T. muris* eggs. The results of both experiments are shown in table II.

The complete failure of the *T. muris* infection in both groups which had been previously infected with *A. tetraptera*, contrasted with the high *T. muris* infection rate in the two groups which were maintained oxyurid free, was highly significant. The worm burdens in the control groups were too low to provide significant contrasts. These results confirmed that infection with *A. tetraptera* reduced the susceptibility of mice to *T. muris* infection.

Experiment 4

Another experiment was performed to investigate the possibility of reciprocal antago-

TABLE II. *Effect of infection with* Aspiculuris tetraptera *on subsequent infection with* Trichuris muris.

Piperazine treatment	*A. tetraptera* and *T. muris* infection	Number of embryonated eggs/mouse	*T. muris* Mouse infection rate	*T. muris* Average infection (range)	*T. muris* Mice harboring > 20 worms	Significance level of contrasts between groups: Mouse infection rate	Significance level of contrasts between groups: Mice harboring > 20 worms	*A. tetraptera* Mouse infection rate
EXPERIMENT 2								
5.11.57	13.11.57	350	0/7	0.0	0	1.0%	> 5.0%	6/7
12.11.57	20.11.57	100		—				
5.11.57	—	—	5/7	12.7	2			2/7
12.11.57	20.11.57	100		(0–39)				
EXPERIMENT 3								
11.2.58	12.2.58	250	0/5	0.0	0	< 5.0%	> 5.0%	5/5
	19.2.58	100		—				
11.2.58	—	—	7/10	13.8	3			1/10
	19.2.58	100		(0–60)				

nism and the importance of the chronological order in which the doses of infective eggs were given to the successful establishment of the two species.

Nine groups of 20 mice were treated with piperazine phosphate and dosed with infective eggs of *T. muris* and/or *A. tetraptera* by a variety of regimens which are summarized in table III.

In this way the mice were cleared of naturally acquired oxyurid infections and maintained oxyurid-free until the time of experimental infection. Group 5 acted as a control for the *T. muris* infections in groups 1, 2, 3, and 4. *A. tetraptera* controls for the same groups were groups 6, 7, 8, and 9 respectively. All survivors were autopsied on day 50. The results of the infections are shown in tables IV and V.

TABLE III. *Regimens of dosage with piperazine phosphate and infective eggs of* Aspiculuris tetraptera *and* Trichuris muris *for mice in experiment 4.*

Group	Day of dosage: Piperazine phosphate as single dose, 443 mg/kg base, by days	Day of dosage: *A. tetraptera* 200 infective eggs/mouse	Day of dosage: *T. muris* infective eggs/mouse
1	1, —, —, —	2	16
2	1, 8, —, —	9	16
3	1, 8, 15, —	16	16
4	1, 8, 15, 22	23	16
5	1, 8, 15, —	—	16
6	1, —, —, —	2	—
7	1, 8, —, —	9	—
8	1, 8, 15, —	16	—
9	1, 8, 15, 22	23	—

TABLE IV. *Antagonism between intercurrent infections with* Aspiculuris tetraptera *and* Trichuris muris *in mice*

Group	Day of infection *A. tetraptera*	Day of infection *T. muris*	Survivors	*T. muris* Mouse infection rate	*T. muris* Average infection (range)	*T. muris* Mice harboring > 20 worms	*A. tetraptera* Mouse infection rate	*A. tetraptera* Average infection (range)	*A. tetraptera* Mice harboring > 10 worms
1	2	16	19/20	8/19	11.0 (0–44)	5	17/19	12.8 (0–46)	11
2	9	16	11/20	2/11	10.3 (0–101)	1	10/11	10.9 (0–28)	6
3	16	16	15/20	8/15	9.9 (0–53)	3	15/15	11.7 (2–25)	8
4	23	16	16/20	11/16	38.1 (0–111)	9	14/16	5.6 (0–31)	2
5	—	16	11/20	10/11	54.7 (0–125)	9	—	—	—
6	2	—	15/20	—	—	—	15/15	18.0 (9–42)	12
7	9	—	17/20	—	—	—	17/17	18.0 (4–35)	13
8	16	—	14/20	—	—	—	14/14	22.4 (1–65)	10
9	23	—	16/20	—	—	—	16/16	18.6 (3–48)	10

TABLE V. *Significance of consrasts between weights of infection in groups from table IV.*

T. muris Mice harboring > 20 worms		*A. tetraptera* Mice harboring > 10 worms	
Groups contrasted	% level of significance	Groups contrasted	% level of significance
1 :5	< 1.0	1 :6	> 5.0
2 :5	< 1.0	2 :7	> 5.0
3 :5	< 1.0	3 :8	> 5.0
4 :5	> 5.0	4 :9	< 1.0
1 :2	> 5.0	1 :2	> 5.0
1 :3	> 5.0	1 :3	> 5.0
1 :4	> 5.0	1 :4	< 5.0
2 :3	> 5.0	2 :3	> 5.0
2 :4	< 5.0	2 :4	> 5.0
3 :4	> 5.0	3 :4	< 5.0

The *A. tetraptera* population developing from the experimental infections is unlikely to have survived unchanged until the time of autopsy, which for group 1 and 6 was 49 days after infection. In groups 6, 7, 8, and 9 high infection rate and worm burden were maintained, presumably due to constant reinfection. Mice in group 5 were free from *A. tetraptera* infection, a condition which may have resulted from the effects of a *T. muris* worm burden, freedom from continuous exposure to infective eggs, or a combination of both these factors. It has been shown that, although groups 1, 2, 3, and 4 were at first exposed to the same dose of *A. tetraptera* eggs, a significantly reduced worm burden was detected in group 4, the weight of infection in the other groups appearing to have been maintained on the same level as in the controls. In this group also, the presence of a heavy *T. muris* infection and/or the absence of exposure to infective eggs would seem to have been of considerable importance in preventing reinfection with *A. tetraptera* during the course of the experiment. This suggests that the pattern of the initial infections was maintained during the experiment and reflected in the infections present at the time of autopsy.

DISCUSSION

Trautmann (1907) reported the suppression of various trypanosome species by intercurrent spirochaete infections. This observation has been confirmed by many workers. It was shown by Schilling (1936) that *Trypanosoma brucei* infection inhibited the normal development of *T. congolense* infection in rodents. Evidence of similar antagonism between intercurrent infections with *Plasmodium* spp. has been presented by Boyd and Kitchen (1937) and several other workers.

Relatively few instances of antagonism between intestinal helminth species have been reported. Cross (1934) described an apparent antagonism between an acanthocephalan, "Neoicanthorinchus sp.," and the cestode *Proteocephalus exigus* in the ciscoe, which he ascribed to a state of non-specific immunity in the host. Larsh and Donaldson (1944) showed that simultaneous infection with *Nippostrongylus muris* reduced the level of infection with *Hymenolepis nana* var. *fraterna* in mice. Recently, Holmes (1957, 1958, 1959) has investigated the antagonism between *Moniliformis dubius* and *Hymenolepis diminuta* in rodents.

From the results of the present series of experiments, it is concluded that antagonism exists between *T. muris* and *A. tetraptera* when both species are present in the same host. The results suggest that the chronological order of the infections is an important factor in deciding which of the species is to enjoy the greater measure of success in its establishment. In all experiments *T. muris* appeared to be extremely sensitive to intercurrent infection, for the worm burden was significantly reduced when *A. tetraptera* has been previously established. A smaller but significant reduction in the worm burden was apparent when simultaneous infection was carried out. In experiment 4 the group of animals in which *A. tetraptera* infection was superimposed on established *T. muris* infection contained fewer *T. muris* at autopsy than did the control group, but the difference failed to reach a significant level. In contrast, this was the only group in experiment 4 in which the *A. tetraptera* burden was significantly affected, though small reductions were recorded from all other mixed infection groups. It is possible to interpret these results as evidence that *A. tetraptera* is more tolerant of adverse conditions produced by competition from intercurrent infections.

In practice ample confirmation of the experimental results has been obtained as, over a period of 2 years since the introduction of piperazine treatment before dosing with *T. muris* eggs, the mouse infection rate in 8,569 animals has been maintained at 83 percent.

The antagonism between *T. muris* and *A. tetraptera* cannot be fully explained, though several different mechanisms could be involved.

It has been demonstrated that mice can acquire an immunity to *T. muris* infection (Shikhobalova, 1939, 1941). Infection with *A. tetraptera* does not seem to stimulate such a reaction in the host, for it has been demonstrated that mice can be successfully reinfected with *A. tetraptera* on the day after a mature infection has been removed by treatment with piperazine (Keeling, unpublished). On this evidence it seems unlikely that an interspecific immune response could be responsible for the reduction in *T. muris* infections, though the possible effect of acquired immunity to *T. muris* on succeeding infections with *A. tetraptera* cannot be dismissed on these grounds.

If acquired immunity is not involved in this antagonism, direct interaction between the parasitic species may be involved. Consideration must be given to at least three kinds of space competition. The first of these is the possibility of mechanical interference. This seems improbable as the microhabitats and behaviour of the two species in the gut show important differences. It is difficult to conceive that *A. tetraptera*, which is a lumen-dwelling parasite, could dislodge *T. muris* when that parasite is established in an intra-mucosal burrow. Two other kinds of space competition which may influence the course of the infections could involve physiological mechanisms. A positive effect might operate through the emanation of materials by one species which prove deleterious to the other by interfering with its normal physiological processes. Such effects could prove particularly important during the establishment of the newly hatched larvae. Similar results might be produced by competition between the species for essential nutrients. This would be of particular importance if the supplies of certain materials were limited and insufficient to meet the needs of both species. Holmes (1959) showed that *Moniliformis dubius* and *Hymenolepis diminuta* competed for a supply of carbohydrates and that abnormalities occurred when the supply of these materials was limited. The mechanisms operating in this instance are under investigation.

SUMMARY

1. Mice carrying natural infections of the mouse oxyurids *Aspiculuris tetraptera* and *Syphacea obvelata* exhibited a low susceptibility to infection with *Trichuris muris*.

2. Removal of the oxyurid burden produced a significant increase in susceptibility to *T. muris*.

3. It was shown experimentally that antagonism existed between *T. muris and A. tetraptera*. This resulted in reduced recoveries of both species from mice carrying mixed infections. The mouse infection rate with *T. muris* was also reduced.

4. The chronological order of infection was shown to be a critical factor. The species established in the mouse first was the more successful. In simultaneous infections, *A. tetraptera* was more resistant to the effects of intercurrent infection.

5. Some possible mechanisms of interspecific antagonism are discussed.

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RESEARCH NOTE

CUTANEOUS MYIASIS IN SMALL MAMMALS.

An unusually high incidence (31.8 percent) of *Cuterebra* infestation among 127 *Peromyscus leucopus* in Ontario, during 1958, was reported by Sealander (1961, J. Mammal. **42**: 57). A similar infestation (27.6 percent) among 214 white-footed mice in Massachusetts, during 1959, is reported by Abbott and Parsons (J. Mammal. In press). These authors remark on the paucity of literature on host species and on the uncertain taxonomic status of the parasites. Their suggestion that all pertinent data be reported prompts this note.

Larval botflies, *Cuterebra* sp. (Diptera: Cuterebridae), have been recorded as dermal parasites in several genera of small mammals by Dalmat (1943, J. Parasit. **21**: 311) and by Test and Test (1943, J. Mammal. **24**: 506). Dalmat found a high incidence of bots during September and October. In my experience they have never been unduly common. My field notes contain data on 1,361 specimens of rodents and lagomorphs of 63 species, collected mostly by trapping. Of these, *Cuterebra* larvae were present in 14 individuals of 8 species, as detailed in table I.

TABLE I. *Small mammals parasitized by* Cuterebra *larvae.*

Species and sex of host		Locality (County)	Date	No. and loc. of bots
Sylvilagus floridanus	F	Madison, Va.	Aug. 1952	1 scapular
" "	M	Rappahannock, Va.	July 1952	1 anal
Tamias striatus	M	Alger, Mich.	Aug. 1948	2 penial
" "	F	Marquette, Mich.	July 1941	2 inguinal
Peromyscus leucopus	F	Ogemaw, Mich.	Aug. 1948	1 inguinal
" "	M	Orange, N. Y.	Aug. 1935	2 penial
" "	F	Albemarle, Va.	July 1952	2 inguinal
" "	M	Madison, Va.	Aug. 1952	1 scrotal
" "	M	Warren, Va.	July 1952	1 scrotal
Peromyscus truei	F	Valencia, N. M.	May 1939	2 inguinal
Peromyscus nasutus	M	Valencia, N. M.	May 1939	1 scrotal
Clethrionomys gapperi	F	Gaspé Sud, P. Q.	Aug. 1959	2 inguinal
Microtus pennsylvanicus	M	Keweenaw, Mich.	Aug. 1941	1 auxillary
Napaeozapus insignis	F	Gaspé Sud, P. Q.	Aug. 1959	1 pectoral

My collecting has been done at all seasons, but principally in the summer months. The earliest cases of myiasis were noted in May, and these in New Mexico specimens. All others were in July and August. In cases where two larval bots occurred on one host, the bots were always in close proximity. Although several hundred *Blarina* and *Sorex* have also been collected, I have never observed larval bots on them.—RICHARD H. MANVILLE, *U. S. Fish and Wildlife Service, Washington 25, D. C.*

EXPERIMENTAL TRICHURIASIS. II. ANTHELMINTIC SCREENING AGAINST *TRICHURIS MURIS* IN THE ALBINO MOUSE

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The global incidence of trichuriasis was estimated at 355 million cases by Stoll (1947). At present the only anthelmintic which has proved relatively effective against *Trichuris trichiura* is the cyanine dye, dithiazanine (Frye et al, 1957; Schwartzwelder et al, 1957). The emergence of this compound is a considerable advance in the chemotherapy of trichuriasis, but some undesirable features are associated with its use. These are side effects, such as nausea and vomiting, the blue-green color of the drug which causes staining of the faeces, and the multiple-dose regimen necessary for cure.

For many years the search for efficient trichuricides has been hindered by the lack of a test organism suitable for large scale empirical screening of drugs. The trichostrongylid nematodes of rodents, *Nippostrongylus muris* and *Nematospiroides dubius,* have played an important part in the evaluation of the activity of bephenium (Copp et al, 1958). Indications given by those primary screening organisms have since been confirmed against a wide range of nematode species of medical and veterinary importance (Rawes and Scarnell, 1958, 1959; Burrows, 1958a, 1958b; Goodwin et al, 1958; Young et al, 1958; Nagaty and Rifaat, 1959; Rubin, 1960; Armour and Hart. 1960). The oxyurids *Aspiculuris tetraptera* and *Syphacea obvelata* in the mouse have been used as indicators of activity against ascariasis and enterobiasis in man (Wells, 1951, 1952; Chan, 1952; Hsieh, 1952; Standen, 1953; Brown et al, 1954). In preliminary work with dithiazanine, McCowen et al, (1957) used oxyurids in mice and then proceeded to confirmatory trials against *Trichuris vulpis* in the dog. Although screening against the mouse oxyurids led to the development of a trichuricide in that instance, those species cannot be regarded as particularly suitable indicators of specific trichuricidal activity.

Following the successful establishment of *Trichuris muris* in albino mice at this laboratory, the high rate of mouse infection (Keeling, 1961) has permitted the use of this parasite in a primary trichuricide screen. This report describes the screening methods employed and the results obtained from tests with a number of reference anthelmintics.

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MATERIALS AND METHODS

Mouse and Parasite Strains

All mice used in the screen are albinos of a single strain (Clarke) weighing 18 to 20 g. The choice of a suitable strain of mice is of considerable importance since two of the six mouse strains tested proved resistant to infection with *T. muris.* The mice are maintained in galvanized metal boxes, fed on a cube diet, and supplied with water ad lib. The strain of *T. muris* (E.R.) is that described by Keeling (1961).

Preparation and Infection of Mice

The mice are prepared for infection by treatment with piperazine to remove naturally acquired oxyurid infections. It has been demonstrated that this treatment significantly increases susceptibility to *T. muris* infection (Keeling, 1961). The mice receive 2 doses of any suitable piperazine salt by gavage, at a dose level equivalent to 443 mg per kg of piperazine base. The doses are spaced 1 week apart. Infective doses of embryonated eggs are administered as described by Keeling (1961), infection being performed on the day after the second dose of piperazine. The infective dose is usually 100 embryonated eggs. Larger doses result in massive infections which are unnecessary and hamper counting at autopsy.

Selection of Infected Mice for screening

Eggs appear in the feces of infected animals from 32 days after infection. As the range of individual infections is considerable, with a few mice proving completely refractory to infection, selection of mice for screening is based on the presence of eggs in their feces. An examination is performed between 37 and 42 days after infection. Fresh fecal smears prepared by a standard technique are examined under low magnification and the weight of infection in each animal is estimated from the numbers of eggs which are found. Four grades of infection are recognised: +++, several eggs appearing in each microscopic field; ++, more than five eggs in the whole smear; +, less than five eggs in the whole smear; – ve, no eggs.

The individual mice are marked with stain to show the estimated weight of infection. Only mice in the

two most heavily infected groups are used in screening, because the scanty eggs in an apparent light infection may result from accidental ingestion of non-embryonated eggs passed by heavily infected mice housed in the same box. The worm burden in mice selected for screening, based on average worm burdens in the untreated control group, ranged from 15 to 87 (average 51) in 34 consecutive tests.

Screening Procedure

For screening, the mice are usually divided into groups of eight. The +++ and ++ infections are distributed as evenly as possible between the groups.

Compounds are dissolved, or in the case of insoluble compounds, suspended with a little gum tragacanth, in distilled water. The concentration is adjusted so that the required dose can be administered by gavage in 0.1 ml per 10 g. body weight. Mice are usually autopsied on the second day after the completion of dosing by the methods already described (Keeling, 1961). In all tests a control group of mice is left untreated. Estimation of drug activity is based on the comparison of this group with treated groups. Two methods of estimation are usually employed: Mouse clearance, contrasting the numbers of animals in control and treated groups which are completely free from infection at autopsy; worm clearance, percentage difference between the average worm burdens of control and treated groups.

These two methods have given comparable results consistently.

RESULTS

In order to assess the validity of *T. muris* as a specific screening organism for trichuricides it seemed desirable to subject this nematode to tests with known anthelmintics, a number of which are reputed to be effective against other species of *Trichuris*. The results of these tests are shown in table I.

TABLE I. *Results of tests with reference anthelmintics against* Trichuris muris *in the mouse.*

Compound	Total dose mg/kg	Unit dose mg/kg	Number of doses	Duration of dosing in days	Survivors	Mouse clearance	Significance at 5% level	Worm clearance %	Significance at 5% level
Bephenium hydroxynaphthoate	1,200	400	3	3	7/8	0/7	–	0	–
	3,600	400	9	3	8/8	2/8	–	0	–
n-butyl chloride	3,600	400	9	3	8/8	0/8	–	0	–
Diethylcarbamazine citrate (Banocide)	3,000	1,000	3	3	9/9	1/9	–	31	–
	1,500	500	3	3	8/9	2/8	–	27	–
	1,800	200	9	3	8/8	0/8	–	25	–
1 :8 dihydroxy-anthraquinone	1,500	500	3	3	8/8	0/8	–	3	–
	3,600	400	9	3	8/8	0/8	–	4	–
Dithiazanine iodide (Telmid)	90	20	2	3	3/8	0/3	–	0	–
		10	5						
	55	10	2	3	5/8	0/5	–	17	–
		5	7						
	100	5	20	10	3/4	0/3	–	0	–
Ficin	12,000	4,000	3	3	6/8	6/6	+	100	+
	6,000	2,000	3	3	8/8	4/8	+	88	+
	3,000	1,000	3	3	8/8	1/8	–	48	–
	6,000	6,000	1	1	8/8	5/8	+	83	+
	6,000	2,000	3	3	8/8	5/8	+	97	+
	6,000	1,000	6	3	8/8	1/8	–	45	–
Hexachloroethane	3,000	1,000	3	3	9/9	0/9	–	0	–
	1,500	500	3	3	8/8	1/8	–	39	–
	3,600	400	9	3	8/8	0/8	–	44	–
Hexylresorcinol	1,000	100	10	5	8/8	0/8	–	0	–
	1,800	200	9	3	4/8	1/4	–	65	–
Oil of chenopodium*	0.2 ml.	0.1 ml.	2	2	4/8	0/4	–	0	–
Phthalofyne (Whipcide)	4,800	1,600	3	3	7/9	0/7	–	31	–
	2,400	800	3	3	9/9	1/9	–	8	–
	2,800	400	6	3	7/8	0/7	–	39	–
		200	2						
Piperazine phosphate	6,000	2,000	3	3	8/8	0/8	–	0	–
	18,000	2,000	9	3	8/8	0/8	–	0	–
Piperazine stearate	6,000	2,000	3	3	8/8	0/8	–	0	–
	15,000	2,000	6	3	6/8	1/6	–	20	–
		1,000	3						
Santonin	180	20	9	3	7/7	1/7	–	53	–
	60	20	3	3	8/8	0/8	–	0	–
Tetrachloroethylene B.P.*	3.0 ml.	1.0 ml.	3	3	8/8	0/8	–	32	–
	11.0	2.5	2	3	5/8	0/5	–	60	–
		1.0	6						
Vermizym	6,000	2,000	3	3	8/8	6/8	+	92	+
	3,000	1,000	3	3	9/9	0/9	–	38	–

* Dose expressed as ml/kg.

The proteolytic enzyme, ficin, was the only substance used which produced a complete eradication of the parasite. The dose, 4,000 mg per kg daily for 3 days, was close to the limit of mouse tolerance. A total dose of 6,000 mg per kg given as a single dose or 3 doses of 2,000 mg per kg, gave a consistently high clearance, but the same total dose as six 1,000 mg per kg doses was less effective. Vermizym*, a compound which contains as its active principle the proteolytic enzyme papain, also showed high activity at a total dose of 6,000 mg per kg given as three daily doses of 2,000 mg per kg. Bephenium hydroxynaphthoate, n-butyl chloride, diethylcarbamazine citrate (Banocide**), 1:8-dihydroxyanthraquinone, dithiazanine iodide (Telmid***) hexachloroethane, hexylresorcinol, oil of chenopodium, phthalofyne (Whipcide†), piperazine phosphate, piperazine stearate, santonin, and tetrachloroethylene did not show significant activity when administered by a variety of dose regimens.

DISCUSSION

In our experience the piperazine treatment given in the preparation of mice for infections has proved adequate to remove naturally occurring oxyurid infection, but it is possible that some modification of this regimen may be necessary in dealing with other mouse and parasite strains. For example, Sheffield et al (1959) found that multiple doses were more effective than a large single dose of piperazine phosphate against oxyurids in the mouse. Selection of mice for screening by fecal examination is necessary when some animals may prove resistant to infection, as the appearance of worm-free mice in the control group is a considerable hindrance in assessing drug activity in treated groups. The method used for grading the infected mice gives a reasonably accurate indication of the worm burden, for it has been found that reexamination of lightly infected and apparently uninfected mice rarely provides more infected animals suitable for screening.

Various dosing regimens have been used in tests with reference anthelmintics. The results of the tests with ficin suggest that a minimum dose of the compound must be given in order to obtain a good effect. Splitting of the dose resulted in reduced activity. No non-enzymic compound was sufficiently active to allow similar variations of dosing regimens to be compared. In routine screening of synthesized compounds the treatment is now given three times daily for 3 days, the dose level being the maximum that the mouse will tolerate. Investigations on the effectiveness of drugs given in food have not yet been performed.

Attempts to assess the validity of *T. muris* as a screening organism have produced some interesting but inconclusive results. The two proteolytic enzyme preparations tested showed activity at high dose levels. Both have produced variable results as anthelmintics in man and other animals. The activity of ficin against whipworm is well known. This enzyme is the active constituent of léche de higueron which has been used extensively in South America against *T. trichiura* infections. Boch (1954) has reported the activity of Vermizym against a number of nematode species in vivo and in vitro. Among the non-enzymic anthelmintics which had no significant effect on *T. muris* were several which had exhibited activity against other whipworm species. The activity of dithiazanine iodide against *T. vulpis* and *T. trichiura* has already noted. Phthalofyne has been used with considerable success in the treatment of *T. vulpis* infections in dogs (Ehrenford, 1954; Burch, 1954; Magrane, 1954), but tests against *T. trichiura* in man produced unsatisfactory results (Hoekenga, 1956; Telfer et al, 1958). Piperazine adipate was reported active against *T. trichiura* (Dunn, 1955), but other workers (Hucker and Schofield, 1956) failed to confirm this. Gordon has found 1:8-dihydroxyanthraquinone effective against *T. vulpis* (1954) and *Trichuris* spp. in sheep (1957). Successful treatment of *T. vulpis* infections in dogs with n-butyl chloride has been described by several workers (Blakely, 1950; Whitney and Whitney, 1954; Whitney, 1957).

The failure of reference anthelmintics against *T. muris* suggests that some important physiological differences may occur between the species of *Trichuris*. Further complications may be found in differences of drug absorption and metabolism in the host. Such considerations, however, cannot constitute the final assessment of the suitability of a primary screen. Among

* Manufactured by Mack, Illertissen, Germany.

** Manufactured by Burroughs Wellcome and Co. (The Wellcome Foundation Ltd.), London.

*** Manufactured by Eli Lilly and Co. Ltd., Basingstroke, England.

† Manufactured by Pitman-Moore Co. Division, Allied Laboratories Inc., Inidianapolis.

existing small animal nematode screens only the murine oxyurids have yet proved completely analogous to the related objective species in the range of their chemotherapeutic susceptibilities. On the other hand, certain species of small animal nematodes are suited to act as indicators of activity for particular types of chemical structure against a related group of objective species. A good example of this is seen in the successful use of *Nippostrongylus muris* as a primary test organism for the bephenium compounds, where indications of activity have been confirmed against related trichostrongyles in sheep. Other compounds, of the phenothiazine and organophosphorus types, show considerable activity against trichostrongyles in sheep, but are not particularly effective against *N. muris* (unpublished observations). In this context *T. muris* is seen as a species which can be maintained in small animals as an organism for the primary screening of potential trichuricides. Its suitability can only be measured when a compound which shows activity against *T. muris* is taken to trial against other trichurid species.

SUMMARY

1. Methods are described for testing compounds against *Trichuris muris* in the mouse.

2. Tests against this organism were performed with bephenium hydroxynaphthoate, n-butyl chloride, diethylcarbamazine citrate (Banocide), 1:8 diphydroxyanthraquinone, dithiazanine iodide (Telmid), ficin, hexachloroethane, hexylresorcinol, oil of chenopodium, phthalofyne (Whipcide), piperazine phosphate, piperazine stearate, santonin, tetrachloroethylene B.P., and Vermizym. Only ficin and Vermizym were significantly active.

3. The validity of the *T. muris* screen is discussed in the light of these results.

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STANDARDIZATION OF TECHNIQUES FOR INFECTING MICE WITH *TOXOCARA CANIS* AND OBSERVATIONS ON THE NORMAL MIGRATION ROUTES OF THE LARVAE*

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Two migratory pathways taken by ascarid larvae in white mice were described by Sprent (1952). Larvae of *Ascaris lumbricoides* and *Parascaris equorum* were found to pass from the intestine through the liver and heart to the lung and then back to the intestine by way of the trachea and esophagus. Larvae of *A. columnaris, A. mustelarum, Toxocara canis, Toxascaris leonina* and *Toxascaris transfuga,* on the other hand, did not take the tracheal route back to the intestine, but distributed themselves from the lung by way of the systemic circulation throughout many of the somatic tissues.

The percentage of inoculated larvae of *T. canis* that later could be recovered from the tissues of mice has varied greatly. In studies reported in 1952 and 1953, Sprent was able to recover 16 percent and 26.8 percent; Ishii (1959) could recover only 10.4 percent and Lee (1960) achieved a recovery rate of 60 percent. It is difficult to ascertain the extent to which technical error was responsible for the wide discrepancies in these results.

In the present study, six factors which may affect the larval recovery rates have been considered. These factors are: (1) age of eggs in the inoculum, (2) adhesion of eggs to the containers and injecting apparatus, (3) inaccuracy of estimates of infective eggs in the inoculum, (4) number of eggs inoculated and volume of the inoculum, (5) methods and apparatus employed in the inoculations, and (6) preparation of the mice to be inoculated. After these several factors had been investigated and the inoculation procedures standardized on the basis of the results, observations were made on the normal migration routes of *T. canis* larvae in mice.

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MATERIALS AND METHODS

Eggs were obtained from the uteri of adult *T. canis,* treated with 1 percent sodium hydroxide to remove tissues and mucus, and incubated in 0.5 percent formalin at room temperature for periods ranging from 24 to 105 days. Unless otherwise indicated, eggs used for inoculation were not older than 2 months. Prior to inoculation the eggs were washed several times with tap water.

The inner surfaces of all glassware, needles, syringes and tubings which came into contact with the inoculum were coated with a silicon compound (Siliclad, a water soluble silicon, Clay-Adams, Inc. New York) to prevent adhesion and the consequent loss of eggs.

For estimation of eggs in the inoculum, a suspension of eggs was shaken vigorously and immediately a 0.05 ml sample was removed with a calibrated 0.1 ml pipette. The sample was placed on a special counting slide marked off in 2.5 mm squares and the number of fully embryonated eggs was counted under suitable magnification. Samplings were repeated at least 10 times and the homogeneity of the suspension at the moment each sample was taken was evaluated statistically. (The poisson variate X, the number of eggs in each sample, is approximately $N(\lambda, \lambda)$. Applying the square root transformation, $Y_i = \sqrt{X_i}$ is approximately $N(\sqrt{\lambda}, \frac{1}{4})$, so that $s^2 = 4 \sum_{i=1} (Y_i - Y)^2$ is approximately X^2_{n-1}. Consequently the homogeneity of the suspension may be checked by comparison of the observed s^2 with the X^2_{n-1} distribution.)

Just before inoculation, a portion of the stock egg suspension was removed, washed free of formalin, and resuspended in water at a density of approximately 1,000 eggs per ml. Using the sampling technique outlined above, volumes of inocula were computed which contained 500 eggs. According to the experience of Lee (1960), 500 infective eggs of *T. canis* constitute the maximum sublethal dose for mice weighing approximately 30 g. In the present studies, female Swiss mice were used in all of the experiments.

To recover the larvae from the tissues, Sprent's (1952) method, slightly modified, was used. The carcass and brain were split into right and left halves and one half was used for counting.

Larvae in the brain were counted directly by squashing the tissues between two glass slides. The carcass and other organs, suspended in digestive fluid (1 percent pepsin pH 1.5), were ground in

a Waring blendor. The amounts of fluid used for digestion were 90 ml and 60 ml for the half carcass and each of the other organs, respectively. The suspensions of ground tissues were then incubated for 3 to 4 hours at 37 C. Following incubation, the suspensions were well shaken and portions were transferred to centrifuge tubes. Thirty ml of the digested carcass suspension and 15 ml of the other tissue suspensions were spun down and the sedimented larvae were counted. Multiplication factors of 6 and 4 were applied to the counts from the carcass and the other tissues, respectively.

The infectivity of inoculated eggs was determined by examining the liver and lungs of mice sacrificed 44 to 48 hours after inoculation.

RESULTS

Optimum post-infection time for sacrificing mice. Most of the larvae which penetrated the intestinal wall had migrated into the liver within 48 hours after inoculation. Judging from the migratory patterns shown in figures 1 and 2,

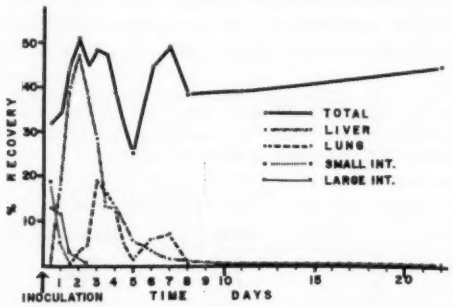

FIGURE 1. Percentage of inoculated larvae of *Toxocara canis* recovered from the intestines, liver and lungs of mice examined at various times during the first 22 days of infection.

the most advantageous time to recover larvae from mice is when they are concentrated in the liver and lungs. Further trials demonstrated that at 44 hours after inoculation approximately 98 percent of the larvae were concentrated in the liver and lungs. In one series of six mice examined at 44 hours after inoculation, the percentages of larvae that were in the liver and lungs collectively were 97.1, 98.2, 97.5, 99.1 and 98.0 (average, 97.6), and in another series of five mice the corresponding percentages were 98.7, 100, 98.6, and 100 (average 98.8). The other organs examined were the carcass, brain, spleen, kidney, heart and intestine.

Infectivity of eggs in relation to age of culture. Two series of observations were carried out with cultures started at different times from different collections of eggs. The longer the period of cultivation beyond 3 months, the lower the infectivity of the eggs and the greater the variance of results (table I). The recovery

TABLE I. *Percentage of* Toxocara canis *larvae recovered from the liver and lungs of individual mice in two series 44 hours after inoculation of 500 eggs of various ages.*

Mouse No.	Pct. of larvae recovered when age in days of inoculated eggs was					
	Series 1		Series 2			
	55	162	24	73	94	104
1	42.0	18.4	51.6	56.4	34.4	26.4
2	45.6	46.4	37.2	40.0	24.0	27.2
3	47.6	52.8	43.2	59.2	31.2	31.2
4	39.2	29.6	47.2	39.2	—	26.4
5	43.4	24.8	48.8	40.0	—	10.4
6	32.4	48.4	40.0	35.2	—	27.2
7	—	44.0	40.8	39.2	—	23.2
8	—	28.0	40.8	46.4	—	19.2
9	—	18.0	—	40.0	—	12.8
10	—	—	—	—	—	27.2
Average	41.7	34.2	44.1	44.0	29.8	23.1
Standard deviation	4.9	12.1	4.9	9.0		5.6

rates at 55 days in series 1 and 24 days in series 2 were almost the same and showed the least variance among the different mice.

Influence of starvation of the mice on the number of larvae reaching the tissues. Three groups of mice were inoculated. One group was inoculated without any starvation at 10:00 a.m.

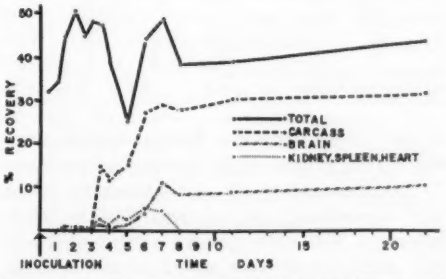

FIGURE 2. Percentage of inoculated larvae of *Toxocara canis* recovered from the carcass, brain, kidney, spleen and heart of mice examined at various times during the first 22 days of infection.

when their stomachs presumably were filled. Food was withdrawn from the other two groups 13 hours and 24 hours, respectively, before inoculation. The mice were killed 44 hours after inoculation and the yields of larvae from the liver and lungs were compared in the three groups (table II). Starvation had no apparent influence on the average total yield, but the standard

TABLE II. *Effect of starvation of mice before inoculation on the recovery of* Toxocara canis *larvae from the liver and lungs 44 hours after inoculation.*

Mouse No.	Pct. inoculated larvae recovered from mice after starvation		
	0 hours	13 hours	24 hours
1	42.0	43.2	45.6
2	45.6	51.2	41.6
3	47.6	35.2	44.8
4	39.2	37.6	44.0
5	43.4	50.4	24.0
6	32.4	32.8	49.6
7	—	—	48.0
Average	41.7	41.6	42.5
Standard deviation	4.9	7.2	7.9

deviations were one and a half times greater in the starved mice.

Effect of body weight of mice on the infectivity of larvae. Two groups of six mice each weighing 20 to 25 g and 31 to 36 g, respectively, were inoculated and examined in the usual manner after 44 hours. No significant difference was observed in the yields of larvae from the two groups (table III). The standard deviation

TABLE III. *Influence of body weight of mice on the recovery of* Toxocara canis *larvae from the liver and lungs 44 hours after inoculation.*

Mouse No.	Pct. of inoculated larvae recovered from individual mice weighing in grams	
	20–25	31–36
1	42.0	31.2
2	45.6	45.6
3	47.6	45.7
4	39.2	53.8
5	43.4	38.4
6	32.4	41.6
Average	41.7	42.8
Standard deviation	4.9	6.8

of the number recovered from individual mice was slightly greater in the group of larger mice.

Effect on infectivity of removing the outer shell membranes from the egg. Fully embryonated eggs were immersed in a mixture of 2 percent sodium hydroxide and 2 percent sodium hypochlorite solutions overnight and washed several times before inoculation. This completely removed the two outer layers, leaving only the thin vitelline membrane. Two batches of eggs treated in this manner were used. The recovery rates from treated eggs were definitely higher than those from normal eggs. However, the normal eggs gave more uniform infections (table IV).

Effect of morphine pretreatment of mice on the infectivity of normal and decoated eggs. fifteen minutes before inoculation, 0.04 ml of 0.5

TABLE IV. *Effect of removing the outer membranes of eggs on the percentage of* Toxocara canis *larvae reaching the liver and lungs 44 hours after inoculation.*

Mouse No.	Pct. of inoculated larvae recovered from mice given		
	Normal eggs	Treated eggs	
		Batch A	Batch B
1	42.0	73.2	71.2
2	45.6	76.4	82.4
3	47.6	52.0	72.0
4	39.2	49.6	46.4
5	43.4	48.0	40.0
6	32.4	92.0	—
7	—	56.8	—
8	—	71.2	—
9	—	74.4	—
Average	41.7	66.0	62.5
Standard deviation	4.9	12.9	16.1

percent morphine sulphate solution was injected intraperitoneally into two groups of mice. One group was given normal eggs, the other decoated eggs. Morphine sulphate treatment apparently increased the number of larvae obtained from normal eggs from 40 percent to 60 percent, but not from decoated eggs (tables IV and V).

TABLE V. *Percent of inoculated* Toxocara canis *larvae recovered 44 hours after inoculation from individual mice treated with morphine before inoculation with normal and decoated eggs.*

Mouse No.	Pct. of larvae recovered when inoculated eggs were	
	Normal	Decoated
1	68.0	62.8
2	52.0	72.9
3	56.0	76.4
4	69.6	72.8
5	57.6	82.4
6	—	60.8
7	—	56.8
8	—	65.6
9	—	53.6
Average	60.6	67.1
Standard deviation	6.9	9.5

The normal migration of T. canis *larvae in mice.* Fifty mice weighing 20 to 25 g were inoculated with *T. canis* eggs simultaneously. Two, three or four mice were then sacrificed and examined at 12-hour intervals for 6 days and on the 7th, 8th, 11th and 22d days following inoculation. In this way the distribution of larvae in the body at various times after inoculation was determined (figs. 1 and 2).

Most of the larvae escaped from the tissues of the small and large intestines 24 hours and 36 hours respectively after inoculation. The highest recovery from the liver was observed at 48 hours. From 48 hours to 84 hours, the number of larvae in the liver decreased rapidly, and

from 84 hours to the 8th day the decrease was more gradual.

Two successive phases of lung invasion by larvae were observed, between the 2d and the 5th, and again between the 5th and the 8th day. Larvae were found in the kidney, spleen and heart from the 2d day to the 8th day. The number in the carcass increased from the 3d day to the 7th day and thereafter remained more or less constant.

Larvae accumulated rapidly in the brain around the 7th day, but after the 8th day there was no apparent increase in numbers.

A most notable feature was a sudden drop in the total recovery rate of larvae after the 4th day and a rise again at the 7th day. It was apparent that some of the larvae were hidden temporarily in other parts of the body than the carcass, liver, lung, kidney, spleen, heart and brain. The digestive tract was not examined after the 3d day because Sprent (1952) had found that the larvae of *T. canis* followed exclusively the somatic type of migration in mice and did not return to the intestine by the tracheal route. To re-examine this question, larvae were carefully searched for in the stomach and intestinal wall of 2 or 3 mice daily as long as any could be found. During the period from the 4th day to the 6th a peak (5.3 percent at the 5th day) of second invasion of larvae into the intestinal wall was clearly observed (fig. 3).

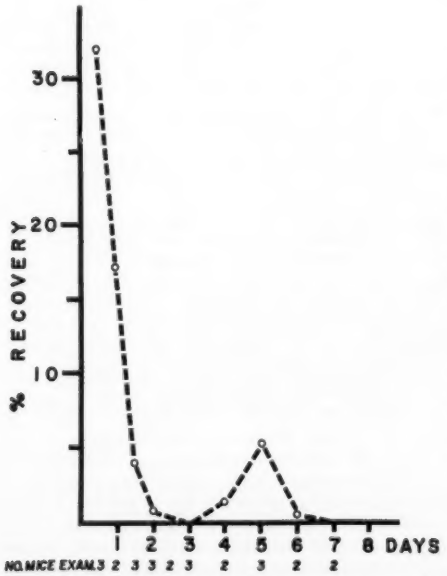

FIGURE 3. Percentage of larvae recovered from the intestinal wall during the first week of infection.

DISCUSSION

The results of inoculation were irregular due to the stickiness of the eggs which caused unknown numbers of them to remain on the walls of the containers, syringe and needle. Attempts to solve this difficulty by thorough washing of glassware and instruments were not successful. However, silicon coating of all contact surfaces almost eliminated this problem.

The drop method for counting eggs in the inoculum has been widely used. Careful measurement of the weight of each drop from the same dropper showed wide variance and irregularity of volume, depending upon the speed of dropping and the stability and position of the dropper. Accurate results of counting were obtained by using a graduated 0.1 ml pipette instead of a dropper-type pipette. When the egg counting was carried out according to the present method, 95 percent of the inocula could be expected to contain between 458 to 546 eggs.

The recovery rate of larvae from the liver and lungs at 44 to 48 hours after inoculation was found not to be a true index of the infectivity of the eggs in the inoculum. There is a difference between the hatching rate and the rate of infectivity of inoculated eggs; however, the differences between these were relatively small, and it was possible to assume in general that the rates of infectivity of eggs, hatching of eggs, and the recovery of larvae from the liver and lungs were essentially the same.

There has been no information concerning the percentage of eggs passing through the intestine without hatching. The results obtained above indicated that the average recovery rate of larvae from normal eggs (without decoating) was between 40 and 45 percent, i.e., more than half of the embryonated eggs appeared to be discharged without hatching in the intestine of mice. If the emptying time of the intestine was prolonged by pretreating the mice with morphine, the recovery rate was increased to more than 60 percent. In other words, 15 to 20 percent more of the eggs hatched if they remained longer in the intestine.

On the other hand, the recovery rate of decoated eggs was only slightly higher than that of normal eggs in morphine pretreated mice. Judging from these observations, it appeared that the maximum recovery of larvae of *T. canis*

in mice was approximately 70 percent, since 30 percent or more of the eggs were unable to hatch in the intestine even when the protein coat was removed or the mice were pretreated by morphine.

Since the emptying time of the intestine appeared to be one of the important factors affecting the recovery rate of eggs, if there were wide differences between the emptying time of the intestine of normal mice and fasted mice, the conditions of feeding would have an important influence on the recovery rate of larvae. Bullough (1948) reported a definite diurnal cycle in the feeding habits of mice. The stomach was filled with food between 9:00 a.m. and 11:00 a.m. and was emptied between 2:00 p.m. and 4:00 p.m. The results of the above experiments showed that there was no difference between the recovery rate of larvae from mice that were normal and those that were fasted before inoculation. The variance of the recovery rate was much smaller, however, in the mice that were inoculated when their stomachs were filled with food. Therefore, the preferred time to inoculate mice was in the morning, and since infectivity of eggs decreased as the age of the cultures advanced beyond 2.5 months, the most uniform results were obtained when the mice were inoculated in the morning (full stomach) with normal (nondecoated) eggs from cultures not over 2 months of age.

Sprent's (1952) observations indicated that migration of *T. canis* larvae in mice is exclusively somatic. However, present data suggested that a small portion of the larvae migrated from the lungs to the intestine, i.e., followed the tracheal route of migration. Evidently the larvae of *T. canis* are able to take both somatic and tracheal routes of migration.

A notable feature of the tracheal migration was the subsequent reinvasion of tissues by larvae after they entered the intestine the second time. This was evident from the slow decrease of the recovery from the liver and the second peak of lung invasion.

Observations by others (Beaver, 1956; Schacher, 1957; Sprent, 1958; and Ishii, 1959) have previously shown that second-stage larvae of *T. canis* in the tissues of mice are infective when fed to other animals including other mice. It is possible that a few of the larvae repeat the tracheal migration one or more times, but this was not detectable in the present experiments.

SUMMARY

The following improvements of technique were developed for the inoculation of mice with *T. canis* larvae: (a) Silicon coating of all inner surfaces of containers and instruments used in the incubation and inoculation of eggs, (b) accurate estimation of the number of eggs in the inoculum, (c) use of eggs stored 2 months or less in cultures, (d) inoculation of mice in the morning when their stomachs contained food, and (e) use of normal (nondecoated) eggs.

It was found that 98 percent of the larvae recovered from mice 44 to 48 hours after inoculation were in the liver and lungs. Yields of larvae from mice inoculated with decoated eggs were greater but more variable than those with normal eggs. Morphine pretreatment of mice increased the yields of larvae from tissues after inoculation with normal eggs, but made no difference in the yields obtained with decoated eggs. The body weight of mice had little influence on the rate of recovery of larvae from their tissues. Although most of the larvae of *T. canis* followed the somatic route of migration, some of them followed the tracheal route, returned to the intestine and re-entered the tissues.

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INFLUENCE OF PREGNANCY AND LACTATION ON MIGRATION OF THE LARVAE OF *TOXOCARA CANIS* IN MICE*

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Ehrenford (1957) reported a marked and increasing resistance to infection with *Toxocara canis* in female dogs 6 to 36 months of age. Schacher (1957) found no growth of larvae beyond the infective stage in the tissues or intestine of non-pregnant dogs, and many larvae were found encapsulated in the liver, kidney, lung, heart, and carcass. On the other hand, Fülleborn (1921), Augustine (1927), Yutuc (1947), Douglas and Baker (1959), and Webster (1958) reported the development of *T. canis* to the adult stage in the intestine of fairly old dogs during the lactation period. Douglas and Baker (1959) kept 5 female dogs free of exposure to *T. canis* for 241 to 358 days after the initial infection and observed the appearance of eggs in the feces of all of them within 25 to 46 days of whelping, and in the feces of the pups in every litter 23 to 40 days after birth. In the old dogs the infections were lost spontaneously after 9 to 108 days of patency.

These findings suggested that pregnancy and lactation influence the migratory behavior and development of *T. canis* larvae in the dog. The present study was undertaken to determine whether gestation and lactation have any influence on the behavior of *T. canis* larvae in mice.

MATERIALS AND METHODS

Methods of cultivation and inoculation of eggs and of recovering larvae from tissues have been described (Oshima 1961). Female Swiss mice, 12 to 20 weeks old, were mated and inoculated with *T. canis* eggs at appropriate times during gestation and lactation. The female mice, caged in groups of three with one male, were examined each morning until mated; days of gestation were counted from the day of vaginal plug formation. Pregnant females were isolated 2 or 3 days before parturition. The litter of each lactating mouse was adjusted to 6 or 7 just after parturition. For a special purpose, litters of some of the mice were withdrawn immediately after birth.

Mice were inoculated at various times during pregnancy and lactation, and the number and distribution of larvae were determined by sacrificing the mice 10 days after inoculation. Some newborn litters were skinned, ground as a whole, digested and examined for larvae.

To determine the effect of hormones (0.1 I.U. of oxytocin, twice a day, 0.05 mg of hydrocortisone acetate, and 0.2 I.U. of prolactin) mice were injected subcutaneously daily for 5 days before and 10 days after inoculation, and were sacrificed and examined for larvae at 2-day intervals after inoculation.

RESULTS

Fifteen pregnant mice were inoculated at various periods ranging from 12 hours to 13 days before parturition. The new-born litter and uterus of the mother were examined just after parturition. No larvae were recovered from any litter and in only one mouse, inoculated 11 days before parturition, were larvae (four) found in the uterus.

The number of larvae recovered from mice inoculated during gestation decreased gradually as the day of inoculation approached that of

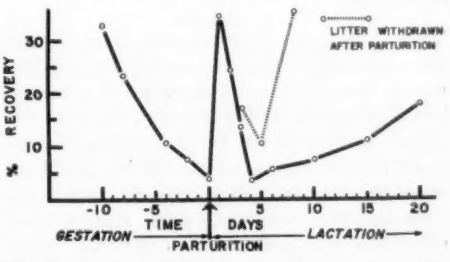

FIGURE 1. Percentage of inoculated larvae of *T. canis* recovered from tissues at 10 days after infection in individual mice that were inoculated at various times during gestation and lactation, and in mice (average of three) with litters withdrawn immediately after parturition.

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parturition. When the mice were inoculated 1 day after parturition, the number of larvae recovered was almost normal. Afterwards, however, the number decreased rapidly and was lowest in mice inoculated on the 4th day to the 20th day of lactation. From mice inoculated on the 4th day to the 20th day of lactation, the recovery of larvae gradually increased (fig. 1).

Nine mice from which litters were withdrawn immediately after birth were inoculated three each on the 3d, 5th and 8th days after parturition and sacrificed 10 days later. Inoculation 3 and 5 days after litter removal resulted in low recovery of larvae, but inoculation after 8 days resulted in recovery of larvae at the normal rate (fig. 1).

Twelve mice were inoculated on the 4th to 6th day of lactation and 2 or 3 of them were sacrificed on the 2d, 4th, 5th, 6th and 11th days of infection (fig. 2). Recovery on the 2d day of

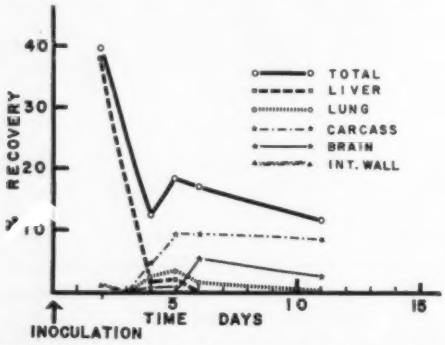

FIGURE 2. Percentage recovery of larvae of *T. canis* from various tissues of mice (average of two or three) that were inoculated 4 to 6 days after parturition and sacrificed 2 to 12 days after inoculation.

infection was the same as that from normal mice (Oshima, 1961), but on the 4th day the rate of recovery dropped sharply and remained low. The number of the larvae in the liver decreased rapidly on the 4th day and none were found in that organ after the 5th day. Numbers recovered from the lung, carcass, and brain were definitely lower than those from normal mice. The intestinal wall was examined carefully but no larvae could be found after the 2d day.

Oxytocin had no effect on the number and distribution of *T. canis* larvae in mice, but hydrocortisone had a mild influence which resulted in a decrease in numbers; prolactin caused an obvious decrease in the rate of recovery of larvae after the 6th day of infection (fig. 3).

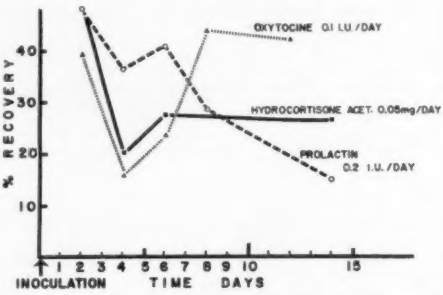

FIGURE 3. Percentage of inoculated larvae of *T. canis* recovered from tissues of mice (average of two) treated daily with lactogenic hormones (prolactin, hydrocortisone acetate, and oxytocin) and sacrificed at various intervals after inoculation.

DISCUSSION

Webster (1956) reported the recovery of second-stage larvae from the litter in all cases when female mice were inoculated during the 1st, 2d or 3d week of gestation. In the present study, those findings were not confirmed. No larvae could be obtained from the litters when mice were inoculated 12 hours to 13 days before parturition. Pike (1960) also failed to find *T. canis* larvae in newborn mice from infected mothers.

Striking differences were noted in the migratory behavior of *T. canis* larvae in normal mice and those in lactating mice. Due to some influence of lactation, fairly large numbers of larvae escaped from the tissues. In normal mice the decrease and subsequent increase in the number of larvae recovered at 4 to 7 days of infection might be due to the second migration of larvae from the lung to the intestine by the tracheal route and from the intestine back to the liver (Oshima, 1961). In lactating mice, however, there was no increase in the rate of recovery of larvae after the initial decrease on the 4th or 5th day, and no larvae were recovered from the intestinal wall after the 3d day. These facts suggest that the larvae which migrated down the alimentary canal after escaping from the lung by the tracheal route could not repenetrate the intestinal wall and were discharged in the feces; i.e., the influence of lactation caused the larvae to lose their ability to repenetrate the intestinal

wall. Possibly, if the host were suitable, the larvae would stay in the intestine and develop to the adult; whereas, if the host were unsuitable, they would be discharged. It is reasonable to presume that the larvae of *T. canis* in the adult female dog can develop to adults in the intestine only during the period of lactation due to this special influence of lactation.

Meites and Turner (1942) observed that the absence of suckling for the 1st week post partum resulted in a fall of the lactogenic content of the pituitary to the ante-partum level. On the other hand, the highest secretory activity of lactation was observed 4 days after parturition (Meites and Nicoll, 1959). Chronologically, these changes in the activity of lactation closely corresponded with the changes observed in the recovery of larvae from lactating mice and those from whom the litters were withdrawn. It is logical to assume that the influence of lactation on the larvae might be due to hormones associated with lactation. Prolactin, oxytocin, and hydrocortisone have been well established as the important lactogenic hormones (Meites and Nicoll, 1959; Bintarningsih et al., 1958; Johnson and Meites, 1958). Among these three hormones prolactin had the most obvious effect on the migration of *T. canis* larvae in mice.

Meites and Turner (1942) found that the prolactin content of the pituitary of rabbits fell as lactation advanced, but decreased at a faster rate if the young were removed. Grosvenor and Turner (1958) observed that prenursing levels of prolactin increased from the 2d to the 6th day post partum then declined towards the end of lactation. These observations suggest a relationship between the secretion of prolactin and the changes in the number of larvae recovered from mice; however, further studies are needed to clarify this relationship.

From the dual fact that the recovery of larvae gradually decreased as the time of inoculation approached that of parturition and there was a sudden increase in the rate of recovery following inoculation on the day after parturition, it is obvious that gestation had a definite influence on the migration of larvae in mice. The sudden increase in the recovery of larvae just after parturition suggests a close relationship to the delivery of the placenta.

SUMMARY

Striking effects of gestation and lactation were observed on the behavior of the larvae of *T. canis* in mice. The percentage of inoculated larvae recoverable from the tissues gradually declined as the inoculations approached the time of parturition, was normal when inoculation was immediately after parturition, again was low when the inoculation was 4 days after parturition, and then gradually increased after later inoculations; when litters were withdrawn at birth the increase in recoverable larvae was greatly accelerated.

The reduction in the rate of recovery of larvae possibly was due to the loss of larvae which took the tracheal route of migration back to the intestine and, failing to repenetrate the intestinal wall, were discharged in the feces. None could be found in newborn mice from infected mothers.

In non-pregnant, non-lactating females the daily subcutaneous administration of prolactin for 5 days before and 10 days after inoculation of larvae had the same effect as gestation on the number and distribution of larvae in the tissues. Hydrocortisone acetate, and oxytocin, similarly administered caused sharp reductions in the numbers of larvae recoverable from the tissues at 4 days after inoculation, but the numbers increased thereafter and, in mice given oxytocin, reached normal levels at 8 days.

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The writer wishes to express his sincere appreciation to Professor Paul C. Beaver for his valuable advice, guidance and interest throughout this study.

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RESEARCH NOTE

THE USE OF EQUI-THESIN AS AN ANESTHETIC IN AVIAN HELMINTH TRANSPLANT STUDIES.

In vivo transplantation studies by the senior author on avian schistosomes and philophthalmids have necessitated the use of a good general anesthetic for chicks. Administration of ethyl ether, pentobarbital or chloral hydrate generally proved unsatisfactory for this purpose.

Gandal (1956, J. Am. Vet. Med. Assoc. **128**: 332–334) induced satisfactory anesthesia of several species of birds, including chickens, with intramuscular injections of Equi-Thesin (Jensen-Salsbery Laboratories, Inc., Kansas City, Missouri). The dosage found to be most effective for chickens was 0.025 ml/g of body weight. We have found intramuscular injections of the anesthetic using the recommended dosage to be effective in operative procedures on newly hatched chicks as well as those up to two weeks old.

Chicks so anesthetized have been used successfully in studies involving implantation of philophthalmids into eyes from which nictitating membranes had been removed, into the coelom and under the neck musculature.—BERNARD FRIED AND JOHN E. BERRY, *Department of Zoology and Entomology and Poultry Science Department, respectively, University of Connecticut, Storrs.*

CANINE MICROFILARIASIS IN EASTERN UNITED STATES

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Comparative geographical surveys of the incidence of *Dirofilaria immitis* infections in dogs in the United States are scanty if not rare. Since the work of Newton and Wright (1956) definitively established the existence of at least two types of microfilariae in the peripheral blood of dogs in this country, previous reports in the literature regarding the distribution of *D. immitis* infections in dogs must of necessity be re-evaluated, with positive identification of the species concerned.

Mudhenk and Greene (1939) reported that of about 200 dogs from Alabama and Georgia 76 percent were positive for microfilariae, while less than 1 percent were positive for adult heartworms upon post-mortem examination. Augustine (1938) failed to find any circulating microfilariae in 30 dogs from New Hampshire, but reported that 8 of 94 dogs (8.5 percent) from Massachusetts were positive for microfilariae of *D. immitis*. Newton and Wright (1957) examined 250 dogs from Maryland, Pennsylvania, and New Jersey and found 18 (7.2 percent) positive for microfilariae, 15 of which had only *Dipetalonema* sp. They concluded that both species probably occur in the south, while in the north *Dipetalonema* is probably common in dogs and *D. immitis* rather uncommon.

Since July 1957, we have examined peripheral blood samples from 600 dogs from Alabama, 841 from Pennsylvania, and 69 from Rhode Island. The present report records the relative incidence of canine microfilariasis in these three areas.

MATERIALS AND METHODS

Blood samples used in this study were obtained from dogs collected in the vicinity of Montgomery, Alabama, Hershey and Pittsburgh, Pennsylvania, and Providence, Rhode Island. Heparin was used as the anticoagulant since oxalate was shown to be responsible for the death of microfilariae within 24 to 48 hours at room temperature (Rothstein and Brown, 1960). All dogs surveyed were over the age of 1 year. No differentiation was made with respect to sex or breed.

Aliquots of heparinized blood samples were examined by the vital staining technique reported by Rothstein (1958), using acridine orange as the fluorochrome of choice, but modified to use the filters as reported by Rothstein and Brown (1960). Two 22 mm × 22 mm coverslip wetmount preparations per slide were examined instead of a single 22 mm × 40 mm coverslip preparation. Differentiation of microfilariae was based on the characteristics noted by Rothstein and Brown (1960). The Chi-square method was used for statistical analysis of the data.

RESULTS

Nine surveys totaling 600 dogs from Alabama were conducted. Two hundred and forty-four samples (40.7 percent) were positive for microfilariae. The microfilariae in the blood samples from the first group of 45 dogs were not differentiated. Twenty-eight of these were positive for microfilariae. The remaining 555 blood samples were differentiated and 6 (1.08 percent) were identified as *D. immitis*, while 210 (37.8 percent) were shown to be *Dipetalonema* sp. Twenty-four surveys comprising a total of 841 dogs from Pennsylvania were also conducted. Forty-three blood samples (5.12 percent) were positive for circulating microfilariae. Of these, 20 (2.38 percent) were identified as *D. immitis*, 21 or 2.50 percent were of the *Dipetalonema* type and 2 (0.24 percent) were of a third type, one distinctly different from the other two.

The average size of this microfilaria was 240 to 250 microns in length by 5 to 6 microns in width; its undulatory movements, and terminal staining with the corroborative coriphosphine O technique (Rothstein and Brown, 1960) within 5 to 10 minutes and absence of the umbrella handle appearance with the modified Knott method (Newton and Wright, 1957) resembled *D. immitis*, while the incidence of one or two per slide was characteristic of *Dipetalonema* infections. Three surveys with a total of 69 dogs from Rhole Island were also conducted. Two (2.9 percent) blood samples were shown to contain microfilariae; one was a *Dipetalonema* infection and the other was a mixed

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infection of *D. immitis* and the third type. The data are summarized in table I.

The incidence of microfilariasis in the Alabama dogs was significantly higher (P = .001) in the Pennsylvania dogs and was almost entirely due to the high frequency of *Dipetalonema* infections in Alabama. The difference between the incidence of microfilariasis due to *D. immitis* in Alabama (1.1 percent) and Pennsylvania (2.4 percent) was not statistically significant at the 0.05 level, but was significant at the 0.1 level. Contrary to popular opinion, our data indicate that within the areas surveyed, the incidence of *D. immitis* may be higher in Pennsylvania than it is in Alabama; further investigation seems warranted.

Within the Pennsylvania sampling, the incidence of *Dipetalonema* infections from Hershey

TABLE I

CANINE MICROFILARIASIS IN EASTERN UNITED STATES

		ALABAMA (MONTGOMERY)	PENNSYLVANIA (HERSHEY)	PENNSYLVANIA (PITTSBURGH)	PENNSYLVANIA TOTAL	Rh ISLAND (PROVIDENCE)	TOTALS
TOTAL	No	600	792	49	841	69	1510
POSITIVES	No	244	39	4	43	2 ⊛	289
	%	40.7	4.9	8.2	5.1	2.9	19.2
DIROFILARIA IMMITIS	No	6 **	20	0	20	1 ⊛	27
	%	1.1	2.5	0	2.4	1.5	1.8
DIPETALONEMA	No	210 **	17	4	21	1	232
	%	37.8	2.2	8.2	2.5	1.5	15.4
OTHER 3rd TYPE	No	0	2	0	2	1 ⊛	3
	%	0	0.2	0	0.2	1.5	0.2

⊛ MIXED INFECTION IN 1 DOG — D. IMMITIS AND 3rd TYPE

** ONLY 555 DIFFERENTIATED; 28 POSITIVES UNDIFFERENTIATED IN 1 SURVEY OF 45 DOGS

TABLE II

SEASONAL INCIDENCE OF CANINE MICROFILARIASIS IN EASTERN UNITED STATES

	ALABAMA				PENNSYLVANIA				
	TOTAL	POSITIVES	D. IMMITIS	DIPETALONEMA	TOTAL	POSITIVES	D. IMMITIS	DIPETALONEMA	OTHER
JANUARY					193	3	1	2	
FEBRUARY					61	2	0	2	
MARCH	81	37	2	35					
1st QUARTER	81	37	2	35	254	5	1	4	
APRIL					248	20	9	10	1
MAY	72	25	0	25					
JUNE									
2nd QUARTER	72	25	0	25	248	20	9	10	1
JULY					100	6	5	1	
AUGUST	45	28	NOT DIFFERENTIATED		50	4	3	1	
SEPTEMBER	79	31	2	29	60	3	1	2	
3rd QUARTER	124	59			210	13	9	4	
	79	31	2	29					
OCTOBER	173	56	1	55					
NOVEMBER					28	1	0	1	
DECEMBER	150	67	1	66	101	4	1	2	1
4th QUARTER	323	123	2	121	129	5	1	3	1
TOTAL	600	244	6	210	841	43	20	21	2
	555	216							

(2.2 percent) was significantly lower (P = 0.01) than the Pittsburgh rate (8.2 percent). However, it should be noted that the Pittsburgh incidence included only 4 cases, a rather marginal statistic. To clarify the differences in these incidence rates, further information should be obtained from these areas.

A comparison of the data from Rhode Island with that of Alabama (table I) indicates that similar conclusions may be drawn. There was a significantly higher incidence of positives in Alabama, due entirely to *Dipetalonema* infections, while there was no difference in the incidence of *D. immitis* in both geographic areas.

The incidence of both types of microfilariasis was of about the same magnitude for Pennsylvania and Rhode Island.

An attempt was then made to note the possible existence of a seasonal variation in the areas tested (table II). There was no seasonal variation in the frequency of positive samples or *Dipetalonema* infections in Alabama. Because of the small number of *D. immitis* cases, an analysis for seasonal variation was not possible.

When the Pennsylvania data were combined and analyzed, a statistically significant seasonal variation at the 0.02 level was found to exist for the frequency of positives. There was no seasonal variation in the incidence of *Dipetalonema* infections but this may not be a valid conclusion due to the small number of *Dipetalonema* cases observed as well as to the fact that at the 0.1 level the data was significant. In contrast to the observations obtained from the Alabama data, there appears to be a seasonal variation in the incidence of *D. immitis* infections in Pennsylvania with significance at the 0.02 level. However, this conclusion may also not be valid because of the marginal number of *D. immitis* cases observed. A conclusion may be drawn from table II that a higher incidence of microfilariasis is prevalent in Pennsylvania during the period from April to September than during the period from October through March.

DISCUSSION

A review of the surveys made in the United States on the incidence of microfilariasis and differentiation of the causative organism reveals that all the work, starting with that of Hinman (1935), up to the report of the presence of a second microfilariid in dogs by Newton and Wright (1956), must of necessity be re-evaluated. Hinman noted that 30 dogs of a group of 93 (32.3 percent) were positive for microfilariae but 7 (or 7.52 percent) did not contain adults. This observation could very well have been one of the earliest indications of the existence of another filariid in dogs in the United States.

The distribution of canine microfilariasis in this country is widespread. Although many lay individuals and even some veterinary practitioners are of the opinion that heartworm is a southern disease, Otto (1949) reported that "infections in disease producing levels are concentrated along the Atlantic Seaboard from New Jersey through Florida and along the Gulf Coast to Texas." That the disease is even more widespread is substantiated by reports from New England (Augustine, 1938; Philips, 1939), from the Mid-west (Otto, 1949) and from the west coast according to comments made by veterinarians to the senior author.

One of the first references to seasonal variation was reported by Ward and Franklin. (1953). They indicated that a higher percentage of positives was noted in dogs examined in the months of March and April in the Clarksdale, Mississippi area. Of 31 dogs examined in 1947, 7 (22.5 percent) harbored microfilariae while 50 of 153 dogs examined in 1951 were positive (32.7 percent). Eyles et al (1954) reported that of the group of 618 dogs examined during a 20-month interval ending in December 1945 in the Memphis, Tennessee, area, 94 (15.2 percent) were positive for microfilariae, with a marked seasonal variation noted; prevalence of microfilariae was greater in the warmer months than during the cool months, with scarcely any infected dogs found during the period of November through February. However, no seasonal variation was found in a sample of 204 dogs examined during a 5-month period ending January 1953, although they noted that the dogs examined in the winter had lower microfilarial and adult worm densities than the dogs examined in the fall.

The results of the present investigation indicate that the incidence of canine microfilariasis in Alabama is about 8 times greater than that found in Pennsylvania and about 14 times higher than in Rhode Island. The incidences of 7.2 percent reported by Newton and Wright (1956) for Maryland, New Jersey, and Pennsylvania, 5.2 percent by Mann and Frata (1953)

for 55 dogs from New Jersey and 33.7 percent by Brown (1939) for 186 dogs from Savannah, Georgia, appear to be corroborated by the present investigation. The extremely high figure of 76 percent reported by Mudhenk and Greene (1939) for 200 dogs from Auburn, Alabama, is about twice as high as our figure for Alabama and may indicate that contrasting incidences may exist in limited geographical areas due to the peculiar ecological factors inherent in those areas. A summary of the geographical incidence of canine microfilariasis in the United States as reported to date is presented in table III. It must be remembered that no differentiation of microfilariae was made prior to 1956 and that the figures prior to that date were given as an index of heartworm disease.

The Alabama incidence reported by us was due almost entirely to *Dipetalonema* infections and this organism might truly be called the filariid of that area. In fact, our data showed

TABLE III. *Geographical incidence of canine microfilariasis.*

Area	Investigator	Dogs	Pct. positive
Alabama	Rothstein, et al., 1960	600	40.7
Pennsylvania	Rothstein, et al., 1960	841	5.1
Rhode Island	Rothstein, et al., 1960	69	2.9
New Orleans, La.	Hinman, 1935	93	32.3
New Hampshire	Augustine, 1938	30	0
Massachusetts	Augustine, 1938	94	8.5
Auburn, Ala.	Mudhenk and Greene, 1939	200	76
Savannah, Ga.	Brown, 1939	186	33.7
New Jersey	Mann and Frata, 1953	55	5.4
Clarksdale, Miss.	Ward and Reeder, 1947	31	22.5
Clarksdale, Miss.	Ward and Franklin, 1953	153	32.7
Memphis, Tenn.	Eyles, et al., 1954	618	15.2
Memphis, Tenn.	Eyles, et al., 1954	204	21.6
Md., N. J., Penn.	Newton and Wright, 1956	250	7.2

that the incidence of heartworm in Alabama was actually less than that of Pennsylvania. Although this difference was not statistically significant at the 0.05 level, it was at the 0.1 level, and the authors feel that further surveys from these areas are warranted to determine the true incidence of Diromicrofilariasis.

The number of cases of *D. immitis* infections precluded any statistical analysis for seasonal variation in Alabama while the data obtained from Pennsylvania indicate that a seasonal variation may exist in the incidence of heartworm infections. While a seasonal variation was also shown to exist in Pennsylvania, there did not appear to be such a variation in the *Dipetalonema* infections. In contrast, there definitely was no seasonal variation of *Dipetalonema* infections in Alabama nor of the frequency of positives from this southern locale.

SUMMARY

Using the previously reported acridine orange vital staining technique for the diagnostic differentiation of microfilariae, 1510 canine blood samples were examined. Of the 600 samples from Alabama, 244 or 40.7 percent harbored microfilariae. Of the 555 differentiated samples, 6 (1.1 percent) were *D. immitis* and 210 (37.8 percent) were *Dipetalonema* sp. Only 43 (5.1 percent) were positive for microfilariae in the Pennsylvania group of 841 dogs. Twenty or 2.4 percent were *D. immitis*, 21 (2.5 percent) were of the *Dipetalonema* type and two (0.2 percent) were of a third type, which was shorter and wider than Dipetalonema but which moved and reacted to the coriphosphine staining technique like *D. immitis*. Of the 69 blood samples from Rhode Island, 2 (2.9 percent) contained microfilariae. One (1.5 percent) was of the *Dipetalonema* type and the other had a mixed infection of *D. immitis* and the third type mentioned above.

No statistically significant difference was found in the incidence of Diromicrofilariasis (heartworm) in the North or South, within the areas surveyed. The incidence of microfilariasis due to *Dipetalonema* in Alabama was about 15 times higher than in Pennsylvania. There was no seasonal variation in the incidence of Microfilariasis in Alabama in contrast to the seasonal variation found in Pennsylvania, with a higher incidence during the period of April to September than through the period from October to March. While there was no seasonal variation in the incidence of *Dipetalonema* infections in Alabama and Pennsylvania, there appeared to be a seasonal variation of *D. immitis* infections in Pennsylvania, with more cases found during the period from April to September. The evaluation of canine microfilariasis in the United States must also consider the existence of a third type of microfilariae.

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BOOK REVIEW

Chandler, Asa C. and Read, Clark P. 1961 INTRODUCTION TO PARASITOLOGY. 10th ed. John Wiley and Sons, Inc., New York. xii + 822 pp., $9.75.

Dr. Read undertook the revision of the 9th edition of this widely used textbook of parasitology after the death of Dr. Chandler in 1958, following Dr. Chandler's established policy of up-dating the subject matter and adding specially pertinent references to the literature and a few useful illustrations. About 14 more pages have been added to the chapters on protozoa and helminths and about 9 more to the chapters on arthropods. The major change in treatment of subject matter has been the elimination of the chapter on spirochaetes and the inclusion of this material in the chapter "Arthropod-borne Organisms Other Than Protozoa." The major improvements are the inclusion of new material in the text and the addition of recent literature citations to the lists at the end of each chapter. It is commendable that Dr. Read has been able to add considerable new information and citations and yet increase the book size by only 23 pages. Doubtless because of the time factor, the recent work of Karmanova (1960) on the life cycle of the giant kidney worm, *Dioctophyma renale*, was not included.

It is hoped that later editions of this excellent textbook will undergo further changes and additions which will be helpful to both students and teachers of the difficult and interesting subject of animal parasitology; e.g., more thorough treatment of (1) parasite classification and nomenclature, (2) parasites of domestic, pet, and wild animals, (3) parasite immunology, physiology, biochemistry and culture, (4) parasitic crustacea and other miscellaneous parasites.—K. C. KATES.

A FIFTH SPECIES OF *NEOECHINORHYNCHUS* (ACANTHOCEPHALA) IN TURTLES

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Neoechinorhynchus emydis (Leidy) was the only acanthocephalan recognized as a parasite of turtles until Cable and Hopp (1954) pointed out that more than one species probably had been confused as *N. emydis,* and described *N. pseudemydis* and *N. chrysemydis.* Their suggestion that still other species may parasitize turtles was confirmed when Fisher (1960) recognized a fourth, *N. emyditoides,* in studying an abundance of material including all that had been recovered from those hosts and deposited in the U.S. National Museum. Included was one lot collected from *Graptemys psendogeographica* by Prof. H. W. Stunkard and deposited in the Museum over 40 years ago. Many of the females in that lot did not agree with any of above species, but because they were contracted and in poor condition due to long preservation in fluid and because eggs removed from some specimens agreed with those of *N. emydis,* the lot was identified as that species but figured separately to show differences (text-fig. 1e, figs. 8 to 10, 19).

Since that paper went to press, biochemical studies in this laboratory have made use of a large quantity of fresh acanthocephalan material from turtles. Because those hosts may harbor mixed infections and males are difficult to identify, those studies were limited to females which are quickly identified to species by the contour of their posterior ends. On one occasion, living acanthocephala were pooled from several *Pseudemys scripta elegans* and a few *Graptemys pseudogeographica* and worms that appeared to be males were removed. Before discarding them, closer examination revealed two specimens that were females containing eggs that were different in structure from those if the four previously recognized species. From study of the living eggs and well extended wholemounts of the two females, it became evident that they and the museum material referred to above represented a fifth species of *Neoechinorhynchus* from turtles.

The male of the new species has not yet been recognized with certainty. Since finding the females, we have examined a number of *G. pseudogeographica* in an effort to find one containing females of the new species only and presumably accompanied by its males, but only *N. emydis* was recovered. For that reason, the following description is limited to the female and egg. Those stages are fairly well illustrated by the figures cited above but additional ones based on the newer material are included. The new species apparently is less common than previously described ones but probably has been overlooked before by us and others because the living female, unless well extended or examined closely enough to detect internal structures, is easily confused with the males of any species with the bursa inverted.

Neoechinorhynchus stunkardi n. sp.
(Figures 1–6)

Diagnosis (all measurements in millimeters): with characters of genus. Trunk of female curved ventrally, up to 22.6 long, 1.182 in maximum width at about level of first ventral nucleus of subcuticula, then tapering gradually until reaching caudal swelling terminating with conical papilla. External praesoma 0.18 to 0.20 long, 0.16 to 0.17 wide. Proboscis hooks in 3 circlets of 6 hooks each, arranged quincunxially. Lateral hooks of anterior circlet 0.079 to 0.083, posterior to others of that circlet which measure 0.066 to 0.073 long. Hooks of middle circlet 0.044 to 0.063; lateral hooks of basal circlet 0.029 to 0.031, others 0.044 to 0.046. Proboscis receptacle 0.437 to 0.474 long, 0.163 to 0.178 in maximum width. Left lemniscus binucleate, longer than uninucleate right one. Terminal female apparatus sigmoid in lateral aspect; mouth of uterine bell 0.666 to 0.770 in straight line from tip of posterior papilla; uterus exclusive of bell and selector apparatus 0.252 to 0.282 long, 0.107 to 0.139 wide; vagina 0.107 to 0.153 long, 0.058 to 0.065 wide; genital pore on ventral slope of caudal papilla. Fully formed eggs, living or preserved in formalin, 0.024 to 0.026 long, 0.015 to 0.017 wide;

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inner shell with few (usually 4) minute, bead-like excrescences, subequatorial in position. Acanthor 0.021 to 0.022 long, 0.006 to 0.007 wide.

Host: The false map turtle, *Graptemys pseudogeographica* (Gray).

Locality: Illinois (upper Mississippi watershed).

Deposited material: Holotype (female) No. 39071 and lot No. 50946, Helm. Coll. U.S.N.M.

Among the species of *Neoechinorhynchus* from turtles, *N. stunkardi* is unique in that the posterior end of the female is swollen and bears a conical, terminal papilla. Furthermore, the terminal female apparatus is sigmoid, even in well-extended specimens, whereas it is straight or almost so in other species. The eggs of *N. stunkardi* are most like those of *N. emydis* in shape and size but in structure are more like eggs of *N. emyditoides* which, however, are longer, narrower, and have short, wide "tubes" connecting two internal shell membranes instead of minute, bead-like excrescences on what evidently is the innermost membrane.

The so-called reversal of the female duct apparatus in the genus *Neoechinorhynchus* was stressed by Meyer (1933) who described a dorsal opening in the selector apparatus, functioning apparently to return incompletely formed eggs to the dorsal ligament sac. We have observed that numbers of ovarian masses as well as eggs are taken into the uterine bell and that with its movements the masses at least escape through a ventral opening at the junction of the bell with the selector apparatus. Whether there is also a dorsal opening as Meyer described was not determined; no eggs were seen to escape at that point. Althongh the opening that was observed is ventral in position, the masses pass through it into the dorsal ligament sac because that sac surrounds the duct apparatus posterior to the mouth of the uterine bell.

The female and egg of *Neoechinorhynchus stunkardi* n. sp. are described from *Graptemys pseudogeographica* in the upper Mississippi drainage. The species is diagnosed by the bulbous posterior end of the trunk, terminating in of conical papilla with the genital pore on its ventral slope. The egg is similar to that of *N. emydis* in size and shape, but more like the egg of *N. emyditoides* in structure.

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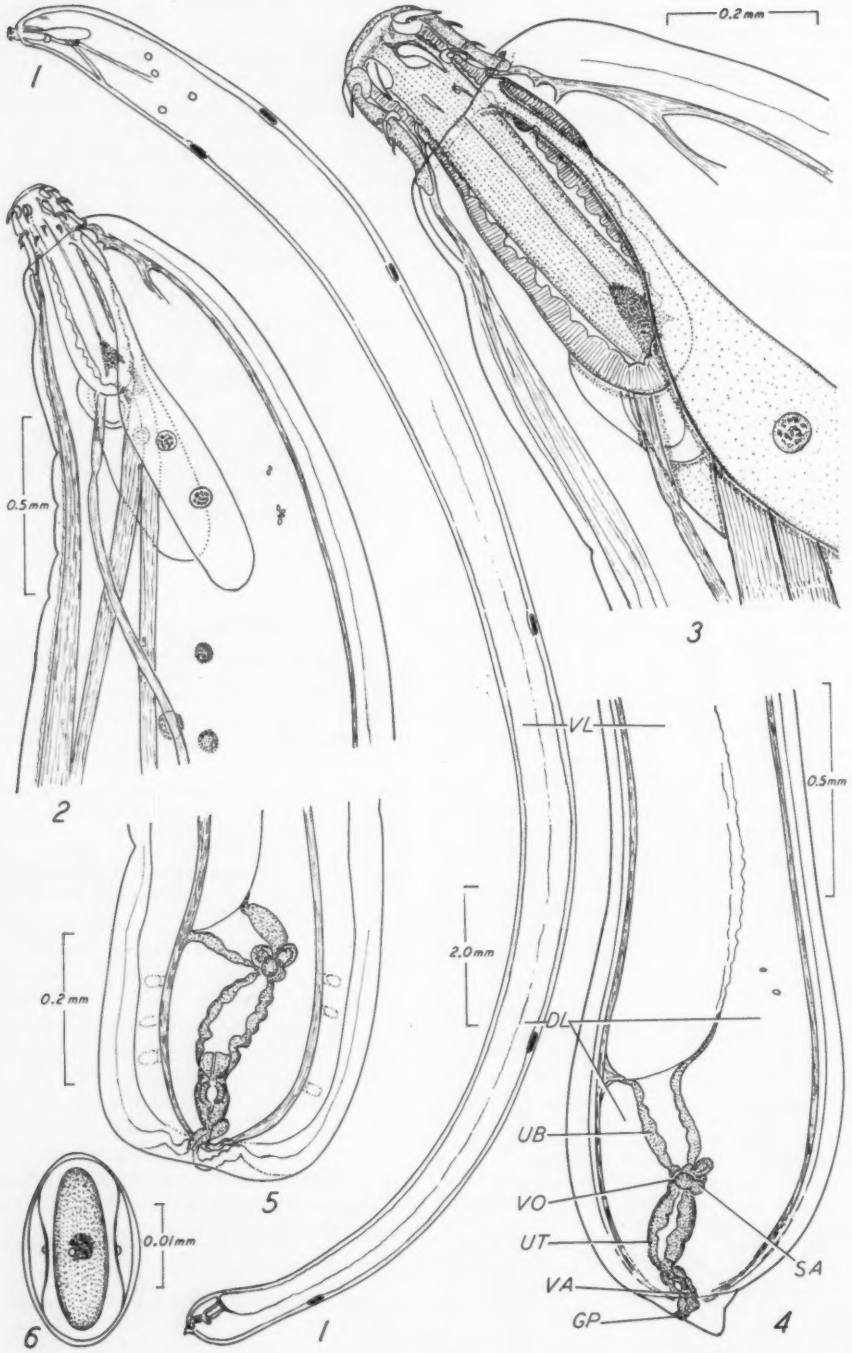
EXPLANATION OF PLATE

All figures concern *Neoechinorhynchus stunkardi* n. sp. and were drawn by microprojection. Abbreviations: DL, dorsal ligament sac; GP, genital pore; SA, selector apparatus; UB, uterine bell; UT, uterus; VA, vagina; VL, ventral ligament sac; VO, ventral opening.

FIGURE 1. Female (holotype entire, side view).
FIGURE 2. Same, anterior end enlarged.
FIGURE 3. Same, further enlarged.
FIGURE 4. Same, posterior end enlarged.
FIGURE 5. Posterior end of contracted female.
FIGURE 6. Egg (for photomicrograph see textfigure 1e, Fisher, 1960).

PLATE I



THE DEVELOPMENT OF ORGAN SYSTEMS IN NEMATOTAENIID CESTODES. I. EARLY HISTOGENESIS AND FORMATION OF REPRODUCTIVE STRUCTURES IN *BAERIETTA DIANA* (HELFER, 1948)

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Most accounts of histogenesis in adult cestodes appeared in the literature before 1935. These include the studies of Young (1908–1935) in *Taenia serrata* and Child (1907 and 1911) on *Moniezia benedeni* and *M. expansa*, Johnstone (1911) on *Tetrarhynchus erinaceus*, Vogel (1929 and 1930) on *Dibothriocephalus latus*, Wisniewski (1930) on the genus *Archigetes*, and Michajlow (1932) on *Triaenophorus nodulosus*. One thorough account (Balss, 1908) was limited to the formation of sex ducts in *Anoplocephala magna* and *A. perfoliata*. In none of these works, however, has an attempt been made to determine the cellular origin of each tissue component in mature and gravid proglottids. In particular, morphogenetic movements have not been analyzed.

In the present series of papers, an attempt is made to identify primitive cell types near the anterior end of the adult strobila and to trace their development in the formation of reproductive and somatic structures of developing proglottids. Also processes of gametogenesis, fertilization, and early embryology are considered.

Since some of the terms employed here are new or possibly ambiguous, the following are defined: adult cestode, the sexually reproducing form in the life cycle of a cestode; anterior end, the end of a cestode with a holdfast; primary anlage (figs 1, 8 and 16 to 19), a longitudinal streak of cells near the anterior end of the strobila which is presumed to give rise to all somatic and reproductive structures in mature and gravid proglottids; in cross sections of *B. diana* this anlage appears as an oval immediately posterior to the scolex, but otherwise appears as a figure "∞" with a thickened center; the thickened central portion of this anlage will be referred to as the central anlage while the remainder (more peripheral portion) will be termed the peripheral germinal ring (figs. 3, 9 to 15, and 24); central anlage (figs. 8, 9 and 17 to 19), the center of the primary anlage or somatic anlage; this anlage (and the next) becomes resolved into a longitudinal series of cell clusters suggestive of proglottids (fig. 20); somatic anlage (fig. 10), the central anlage after all the gonial cells have left it; peripheral germinal ring (figs. 3, 9 to 15 and 24) the peripheral ring of cells (in three dimensions, a hollow cylinder) associated with the primary anlage, somatic anlage, or posterior to either of these two primordia; this primordium gives rise to somatic cells only; Fuhrmann referred to it in 1895; somatic generative cell (figs. 15, 28 and 29), one of the two basic cell types in the primary and somatic anlagen; this cell seems to be the forerunner of all posterior somatic structures; the other primitive type is gonial (figs. 15, 28 and 29) and is the precursor of oogonia, spermatogonia, and yolk cells.

MATERIALS AND METHODS

Altogether, sections of over 100 specimens of the nematotaeniid cestode, *Baerietta diana* (Helfer, 1948), were studied, but most of the drawings and detailed study were based on 15 sets of serial sections. Six whole mounts were also used in the study. Worms were dissected from the small intestine of *Batrachoseps attenuatus* (a plethodontid salamander), relaxed in chloretone, and transferred to fixative as quickly as possible. Worms were fixed in the reagents of Duboscq-Brasil, Bouin, King (Richards, 1911), Carnoy (6:3:1 and 3:1), and Gilson. For cytological details, sections at 12 microns were stained regressively in Heidenhain's iron haematoxylin, or progressively with concentrated Harris' haematoxylin (Galigher, 1934), and counterstained, if at all, with fast green, orange G, or eosin. For sections showing nuclear detail as well as chromosomes, Feulgen procedures with

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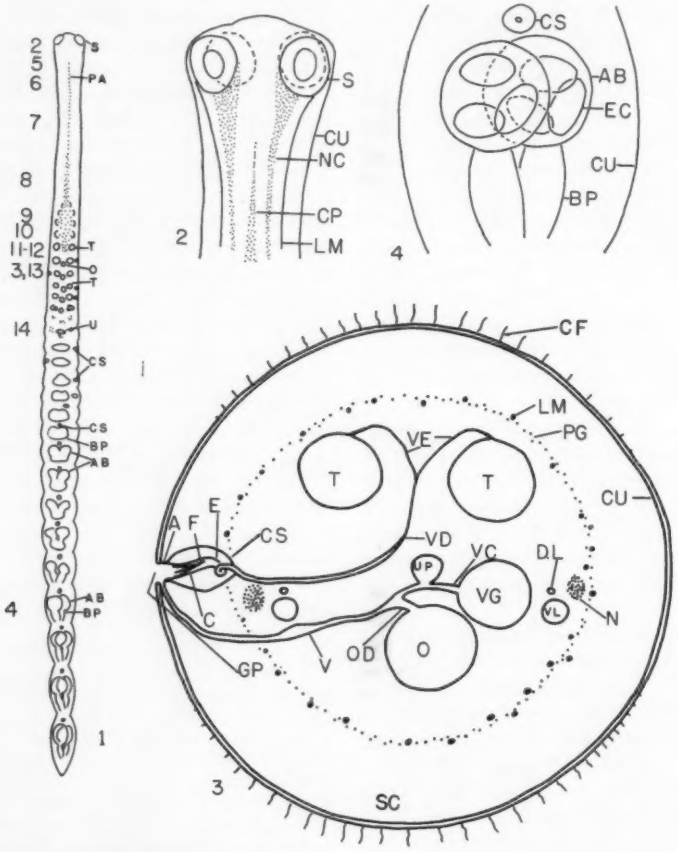
either Schiff's reagent or one of the azure reagents (azure A, azure B bromide, and thionin) were used (DeLamater, 1951). The latter stains were prepared by adding 25 cc of dilute sulfurous acid (distilled water, 200 cc of normal potassium metabisulfite, 10 cc, and 10 cc of normal hydrochloric acid) to 45 cc of 0.25 percent stain. At 60 C the optimum hydrolysis times were 6 minutes for material fixed in Duboscq-Brasil fluid and 8 minutes for material fixed in Carnoy. The azure reagents gave a much needed dark stain, while Schiff's reagent did not stain larger subcuticular nuclei and oocyte nuclei dark enough for careful study. The material stained with Schiff's reagent was counterstained with fast green (0.2 percent in 95 percent alcohol), while sections stained with azure reagents were counterstained with triosin.

Because of its small size (usually shorter than 50 mm) and simple construction (figs. 1 to 4), *B. diana* is ideally suited for morphological studies. Compared with other cestodes the author has examined, the cells of *B. diana* are large and widely spaced so that they can be easily differentiated. For study of embryos, *B. diana* is also valuable. Even the earliest embryos are well separated from each other in the uterus, and the chorion is laid down early enough that polar bodies do not completely escape from developing oocytes. The amount



Figures 1–4 (for abbreviations see figures 5 to 14).

1. Diagram of a whole mount. The suckers, scolex, and different levels of differentiation are shown. The numbers at the left of the diagram refer to the levels represented in the 13 following figures. For clarity this diagram is shorter and thicker than the actual specimen.

2. A scolex. The suckers, muscle fibers, central primordium, and nerve cords are labeled. Compare this with figure 17.

3. A cross section of a mature proglottid (level 13 of figure 1). Notice the circular shape of this section. The stage is also represented in figures 13 and 32.

4. A gravid proglottid. The basal portion of the paruterine organs are partly derived from the uterus.

of globular yolk in the oocytes is small and does not obscure the chromosomes and asters; the egg shell membranes, when embedded in paraffin, do not become hard enough to destroy adjacent tissues.

OBSERVATIONS

The Early Primary Anlage

Immediately posterior to the scolex of *B. diana* the primary anlage appears approximately oval in cross section (fig. 5), but in less than 50 microns posterior to this level, both dorsal and ventral indentations and the center of the neck begins to fill with cells, giving the primary the shape of a figure "∞" (figs. 6, 7 and 17). In an average specimen (about 250 microns in diameter through the neck), the primary anlage measures approximately 60 to 75 microns dorsoventrally, and 150 microns laterally, and is distributed symmetrically about the longitudinal axis of the worm.

The cells located in the early anlage (see the introduction for definitions of terms) are shown in figures 28 and 29 (see also fig. 15). In the interest of clarity they are named here before their identity is established on the basis of evidence. In the actual study of slides, however, the author determined their identity by tracing backward (toward the scolex) from fully differentiated organs and tissues. Two primitive and three derived cell types are visible in the figures cited. One of the primitive types is gonial (GC); it is larger than others (about 10 microns in diameter), its cytoplasm stains darkly, and its nucleolus is conspicuous after haematoxylin (the nucleolus is well focused in the cell indicated by the arrow in figure 28). The other primitive cell type, the somatic generative cell, is the smallest kind visible in figures 28 and 29. Its shape is characteristically fusiform and it measures about 3 × 5 microns (fig. 29). Typically its nucleus is spherical and stains very heavily after haematoxylin and azure A. Its nucleolus, however, is not conspicuous in material stained with haematoxylin. Both primitive cell types are frequently seen in various stages of division (table I). The other three cell types are fully differentiated and have not been observed dividing (table I). They include parenchymal cells, myocytes, and excretory cells. Development of these three cell-types will be considered in a later paper; but it seems appropriate to list here the characteristics which serve to differentiate them from either of the two primitive types. Myocytes are associated with twisted muscle fibrils, the amount of cytoplasm immediately surrounding the nucleus is slight or negligible, and the nuclear membrane is very heavy after haematoxylin. Parenchymal cells are intimately associated with the parenchymal network, have extremely vacuolated cytoplasm associated with the nucleus, or in advanced cells, the cytoplasm associated with the nucleus is almost completely lacking (fig. 15). Excretory cells are easily recognized because they are associated with excretory ducts and canals or contain flames. Finally, identification of the two primitive cell-types is facilitated by their occurring only in the figure "∞" of the primary anlage.

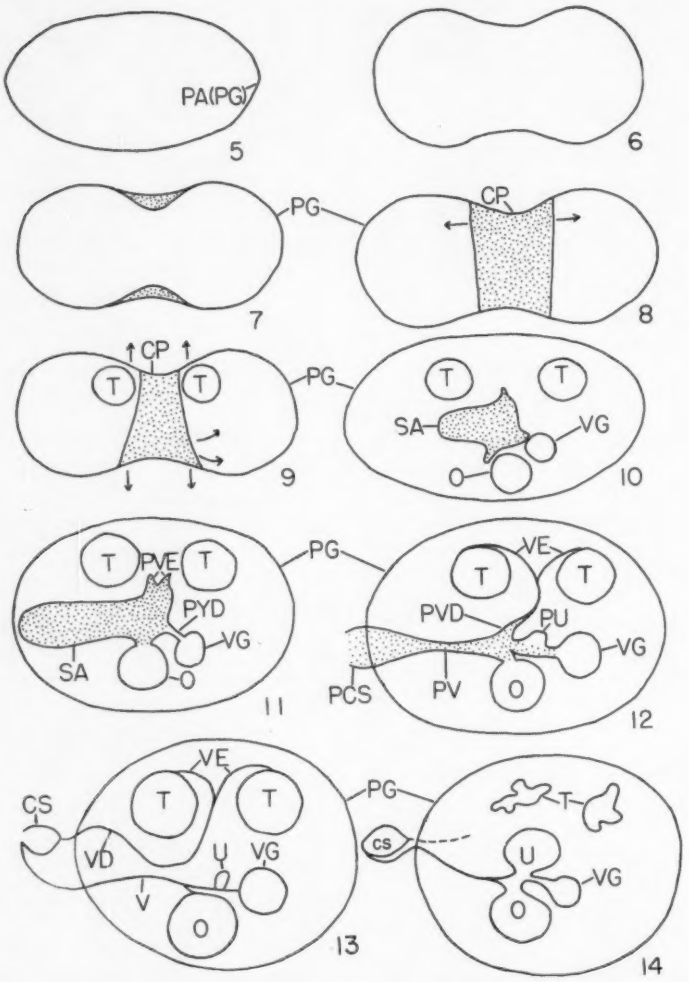
Histogenesis of the Testis, Ovary, and Vitelline Gland

The level of mitotic activity in the primary anlage is relatively high (table I) and soon the region of the central anlage becomes filled with cells (observe the sequence of figures 5 to 8 and 16 to 19) As the central anlage develops it becomes subdivided into a longitudinal series of cell masses suggestive of proglottidization (figs. 1 and 20). The mechanism of proglottidization, and possibly migration of spermatogonia, involves histogenesis of parenchyma which is the subject of a subsequent paper in this series.

TABLE I. *Mitotic indexes in tissues of* B. diana.

(The mitotic index in the different primordial tissues is approximately 3 to 4 percent. In some specimens, however, there are virtually no mitotic figures at all. This suggests that there may be a periodicity similar to that hypothesized by Richards (1911), or possibly that the worms were not fixed soon enough after dissection. The two last (fully differentiated) tissues were selected because of their large, easily counted nuclei, but mitotic figures are also absent in other differentiated tissues; an exception is the wall of the uterus.)

Tissue	No. nuclei counted	Mitotic index
Primary anlage	1926	3.2 pct.
Peripheral germinal ring:		
(1) level of ovarian primordium	532	3.2 pct.
(2) level of early uterus	722	3.2 pct.
(3) level of early paruterine organs	875	4.5 pct.
Subcuticle	2900	0.0 pct.
Parenchyma	3000	0.0 pct.



Figures 5–14. A summary of organogenesis in the reproductive system of *B. diana*. All diagrams represent cross sections of *B. diana* (about 75×). Numbers 5 to 10 are from single sections, while figures 11 through 14 are reconstructed from 4 to 7 sections, each 12 microns thick. See text for explanation. Some of the figures here correspond with plate figures, approximately as follows: 6 with 16; 7 and 8 with 17; 9 with 19 and 20; 10 with 11; and 11 to 13 with 23 to 25.

Abbreviations: A, genital atrium; AB, apical bulb of the paruterine organs; BP, basal portions of the paruterine organs; C, cirrus; CF, cuticular fibril; CP, central anlage; CS, cirrus sac; CU, cutucle; DL, dorsal longitudinal excretory canal; E, ejaculatory duct; EC, egg capsule; F, ejaculatory fibrils; GP, genital pore; LM, longitudinal muscle fiber; N, nerve cord (cross section); NC, nerve cord; O, ovary; OD, oviduct; PA, primary anlage; PCS, presumptive cirrus sac; PG, peripheral germinal ring; PU, presumptive uterus; PV, presumptive vagina; PVE, presumptive vasa efferentia; PVD, presumptive vas deferens; PYD, presumptive yolk duct; S, sucker; SA, somatic anlage; SC, position of the subcuticle; T, testis; U, uterus; UP, uterine primordium; V, vagina; VC, vitelline duct; VD, vas deferens; VE, vas efferens; VG, yolk gland; VL, ventral longitudinal excretory canal.

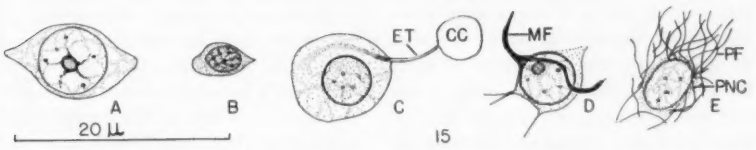


Figure 15. Diagrams of cell types encountered in the primary anlage. All are figured as they appear after Duboscq-Brasil's fixative and Heidenhain's iron hematoxylin.

Abbreviations: A, gonial cell; B, somatic generative cell; C, excretory epithelial cell; D, myoblast; E, parenchymal cell; CC, excretory canal in cross section; MF, myofibrils; PF, parenchymal fibrils; PNC, perinuclear cytoplasm; ET, excretory tubule.

About 75 to 100 microns posterior to the first appearance of the central anlage, the testicular primordia begin to appear. They are first seen as paired clumps of cells budding from the dorsolateral quadrants of the central anlage (figs. 8, 9 and 19 to 21). The exact means by which they are formed has not been determined, but the following two observations suggest that ameboid movement is partly responsible for the migration of gonial cells to the site of the testis: (1) Although gonial cells predominate in the early central anlage, they are not there after the sex glands differentiate (the central anlage in figure 21 is early and contains many gonial cells; in figure 22, however, the sex glands have formed and gonial cells are completely missing from the central anlage). (2) Irregularities in the outlines of early gonial cells are most suggestive of pseudopodia (figs. 21, 28 and 29).

The ovary and vitelline gland appear to be formed in much the same way as the testis. In figure 21 the ovarian and vitelline gland primordia are visible (follow labels O and PVG in the sequence of figures 21, 22 and 23). In figure 21, as is typical, the oogonia are all spindle-shaped and oriented in a direction which is suggestive of ameboid movement toward the future site of the ovary (compare with fig. 3). Since the vitelline gland ultimately develops very near the center of the proglottid, its primordium, which is also near the center (figs. 21 and 3), would hardly need to move. In figure 21, for example, presumptive yolk cells are all rounded and do not appear ameboid.

Sex Ducts and the Somatic Anlage

While the reproductive glands are being formed, the peripheral germinal ring becomes separated from the central anlage (figs. 9, 10 and the sequence 19, 22, 23 and 24). Immediately after the sex glands become arranged in their final positions, cells in the central anlage (equivalent to somatic anlage, because all its cells are now somatic generative) begin to multiply, and in two or three proglottids posterior, a well developed somatic anlage results. Most cells in the somatic anlage move laterally; some reach the cuticle where they become the cirrus sac and genital atrium (figs. 23 to 26), while others do not move so far lateral and give rise to the vas deferens (fig. 25) or vagina (figs. 24, 26, 12 and 13). At first the primordia of the vagina and vas deferens are part of the same laterally-directed cord of cells (figs. 11, 12 and 23); but soon the cord becomes resolved into two, one dorsal and anterior (vas deferens) and the other ventral and posterior (vagina), so that the vas deferens and vagina come to lie 15 to 20 microns apart (figs. 24, 25 and 26). Since none of the frontal sections prepared showed both of these sex duct primordia, it is necessary to show them in separate photographs (compare figures 25 with 24 and 26).

The minor sex ducts (viz, the oviduct, vitelline duct and vasa efferentia) are first evident as branches or buds from the early somatic anlage (figs. 22, 10 and 11). The grosser aspects of their future development is easily followed in the proglottids which succeed those containing the early budding stages (figs. 11 to 13 and 22 to 25). However the mechanism by which these minor sex ducts come to occupy their definitive positions has not been determined with certainty. It appears that the early buds, once formed, merely stay in place, while the main mass of somatic anlage migrates laterally (figs. 11 to 13 and 22 to 25). It is also possible that elongation of the vasa efferentia and vitelline duct together with the vagina and vas deferens is partly due to expanding parenchymal vacuoles.

DISCUSSION

Identification of primitive cell types. The

two primitive cell types in *B. diana* correspond closely with those reported in other cestodes. As far as the author can determine, Prenant (1922) was the first researcher to recognize the existence of two primitive cell types in cestodes. In studying *Hymenolepis diminuta* and *Bothriocephalus microcephalus,* Prenant referred to primitive somatic cells as "cellules fixes" and to primitive gonial cells as "cellules libres." Later Vogel (1929) described corresponding cell types in oncospheres of *Dibothriocephalus latus* (he referred to the gonial type as a "plastin cell"), and Wisniewski (1930) observed the same two types in larvae of *Archigetes.* Wisniewski noted that gonial cells are occasionally transformed into somatic types, a point not confirmed in the present paper because larval stages of *B. diana* have not been discovered.

Before Prenant's work (1922), very few attempts were made to identify cell types responsible for forming reproductive organs, three of these works can be mentioned. The first is that of Child (1907–1911). He felt that reproductive organs of *Moniezia expansa* and *M. benedeni* were elaborated by parenchymal cells and myocytes (as a result of amitosis). Papers cited in the previous paragraph (and also the present one) do not support the conclusions of Child. Also findings with a number of organisms studied subsequent to Child's research would tend to weaken the argument that variations in species account for morphogenetic peculiarities in *Moniezia.* In *B. diana* it is particularly easy to deduce that parenchyma cells and myocytes do not contribute significantly to reproductive structures; these two somatic cell types are quite distinctly different from gonial cells and undifferentiated somatic cells (figs. 15, 28 and 29), the pattern of morphogenesis is relatively simple to follow (see materials and methods), and the adult worm is small enough to permit studies of complete serial sections. The second paper is that of Young (1913). This author, in studying *Taenia pisiformis,* agreed with Child's interpretations and especially supported his conclusions that testes arose from muscle sheath cells, The third is that of Fuhrmann (1895). This author recognized that cells in the peripheral germinal ring of *Nematotaenia dispar* divided frequently and, furthermore, postulated that these cells might give rise to differentiated tissues. The present research, as well as that later in this series, confirms this postulate of Fuhrmann.

Early morphogenetic movements. The writer has found only one previous paper in which there is a suggestion that cellular migrations occur early in adult cestode development; it is the excellent work of Wisniewski (1930). Even in his paper, however, there is only a brief statement pertaining to cell migrations. On page 125 of his text Wisniewski stated that gonial cells of *Archigetes,* which earlier had been dispersed within the central part of the parenchyma, begin to organize themselves in a pattern suggestive of their future fates. Other authors all stated that cell masses form sex ducts in situ. Young (1913), though not certain, postulated that reproductive structures in *Taenia pisiformis* arose in situ from several cell centers, while Richards (1911) and Leuckart (1886) concluded that fewer cell centers were involved. Furthermore, Leuckart (1886), Schmidt (1888), Balss (1908), Gough (1911), and Young (1913) all felt that the vas deferens and vagina arise in situ. Child (1907c), while concurring with this hypothesis, reported "pseudomigration" in the development of the cirrus sac of *Moniezia.* According to Child, resorption of cells at the medial edge of the primordium and proliferation at the lateral edge gave the impression of migration, even though each cell in the primordium remained stationary. In *B. diana* pseudomigration of the cirrus sac anlage does not appear to occur. Disintegrating cells have not been observed at the medial edge of the presumptive cirrus sac, and cell division does not appear to be localized at its lateral edge (fig. 22). Since Child did not differentiate between parenchyma cells and primitive somatic cells, it could be that he mistakenly identified parenchyma cells (uncovered at the medial edge of the cirrus sac primordium during migration) with degenerating cirrus sac cells.

It seems appropriate to emphasize the importance of preparing serial sections for study of morphogenesis in adult cestodes. In the species studied here, at least, it would have been virtually impossible to interpret cellular movements by studying sections taken at spaced intervals.

Examination of table II will serve to illustrate this point. For example, the omission of a piece of worm only 150 microns long could, properly chosen, result in missing any one of the following events: the formation of the central anlage or testicular buds, migration of oogonia, development of the presumptive yolk gland, and the migration of the cirrus sac pri-

TABLE II. *Distance from scolex of development of sex organs.*

Event or structure	Microns (approx.) posterior to scolex
Scolex (anterior margin of suckers)	0
First appearance of primary anlage	25–50
Central anlage develops	100–150
Internal proglottidization begins	350–500
Testicular buds become distinguishable	500–550
Ovary and vitelline primordia first appear	520–570
Cirrus sac primordium first reaches the cuticle	700–950
Mature proglottid with early uterus	3500–4000

mordium. It could easily be that the failure to examine serial cross sections has been the major factor confusing the question of whether morphogenetic movements occur in early cestode strobilae. Even though significant events might be further separated in longer species, there is no a priori guarantee of this, so that authors publishing in this field should exercise extreme care to include all pertinent intermediate stages.

SUMMARY

1. In the nematotaeniid cestode, *Baerietta diana,* nearly all the cellular components of mature and gravid proglottids appear to be derived from a single longitudinal streak of cells located immediately posterior to the scolex—the primary anlage.

Most reproductive organs are formed by cells in the central part of the primary anlage. Dorsal buds from this central part give rise to the testes, ventral buds develop into the ovary and vitelline gland, and cells migrating laterally from it become the cirrus sac, vagina and vas deferens.

2. Within the primary anlage there are two major primitive cell types: one, somatic generative; the other, gonial. The latter cell type is incorporated into the sex glands, including the testes, ovary, and yolk gland. Somatic generative cells (alone in the primary anlage after the gonial cells are gone) then form the reproductive ducts.

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Explanation of Plates

All drawing were prepared with the aid of camera lucida. Unless otherwise indicated, figures and photographs were taken from material fixed in Duboscq-Brasil, sectioned at 12 microns, and stained with Heidenhain's iron haematoxylin.

ABBREVIATIONS: AB, apical bulb of the paruterine organ; CC, longitudinal excretory canal; CP, central anlage; CS, cirrus sac; CU, cuticle; EC, exeretory cell; EMC, early myocyte; EPC, early parenchymal cell; ET, excretory tubule; GC, gonial cell; GSG, growing somatic generative cell; MC, myocyte; MF, myofibrils; O, ovary; OD, oviduct; OO, oocyte; PBP, presumptive basal portion of the paruterine organ; PC, parenchymal cell; PF, parenchymal fibril; PG, peripheral germinal ring; PCS, presumptive cirrus sac; PMC, presumptive myocyte; PNC, perinuclear cytoplasm; POD, presumptive oviduct; PPC, presumptive parenchymal cell; PS, pseudopodium; PT, presumptive testis; PU, presumptive uterus; PV, presumptive vagina; PVD, presumptive vas deferens; PVE, presumptive vasa efferentia; PVG, presumptive yolk gland; PYD, presumtive yolk duct; SA, somatic anlage; SG, somatic generative cell; T, testis; U, uterus; V, vagina; VG, yolk gland.

Plate I

Cross sections of *B. diana* in the neck region up to the level of the earliest presumptive testes. Fixed in Duboscq-Brasil, sectioned at 12 microns, hydrolyzed, and stained with azure-A according to the technique of DeLamater (1951). Refer to figures 1, 3 and 5 to 14 for spatial orientation.

Figure 16. A section immediately posterior to the scolex. The central anlage has not yet developed.

Figure 17. A section about 120 microns posterior to the preceding. Cells have appeared in the center of the primary anlage (primary anlage is equivalent to central anlage plus peripheral germinal ring).

Figure 18. Cross section approximately 150 microns posterior to the one in figure 17. The central anlage is filled with cells. Most of the cells are gonial; they do not stain darkly and their nuclei are relatively large. Compare them with the darker-staining masses of somatic cells located in the dorsal and ventral portions of the peripheral germinal ring.

Figure 19. A level about 100 microns posterior to figure 18. The presumptive testes appear as paired clusters of gonial cells budding laterally from the central anlage. Refer to figures 20 and 21 for details of this budding process.

Plates II–IV

All photographs are of *B. diana*, fixed in Duboscq-Brasil, and sectioned at 12 microns. Material in figures 19, 20 and 26–29 was stained with Heidenhain's iron hematoxylin while sections in figures 22–25 were stained with Galigher's hematoxylin and counterstained with fast green.

Figure 20. Frontal section showing formation of an early testis. The presumptive testis has not developed in the most anterior proglottid of the figure, but it gradually develops at the left hand margin of successive segments of the central anlage. The second proglottid in the figure approximately corresponds to the cross section in figure 19, while the next to last proglottid is at about the level represented in figure 21. The arrow between the last two proglottids indicates the formation of a space between the forming testis and the central anlage.

Figure 21. Cross section showing central anlage as it begins to subdivide into sex gland primordia (compare with figure 19 where only the testicular buds are evident). Gonial cells in the presumptive testes are immediately beneath the two long arrows. The three small arrows indicate somatic generative cells; their small size and dark staining are illustrated.

Figure 22. Section which is seen about 7 to 10 proglottids posterior to the one shown in figure 21. All glands appear more distinct than in figure 21, and the somatic anlage gives the appearance of bulging laterally (toward the reader's right). Many small dark cells (somatic generative) are seen in the somatic anlage. In this and the four following figures, the cirrus sac will develop on the right, while it will develop on the left in figure 21. Therefore figure 21 is a mirror image of figures 22 to 26.

Figure 23. Figure showing the lateral position of the cirrus sac primordium. Also the presumptive oviduct, vas deferens, and vagina are visible. The aporal testis is excluded from the photograph. The section is taken about 10 proglottids posterior to the one in figure 22.

Figure 24. Section of an early mature proglottid primarily to illustrate the cirrus sac adjacent to the cuticle and to show the female sex ducts.

(Explanation of figures 25–29 on p. 680)

PLATE I

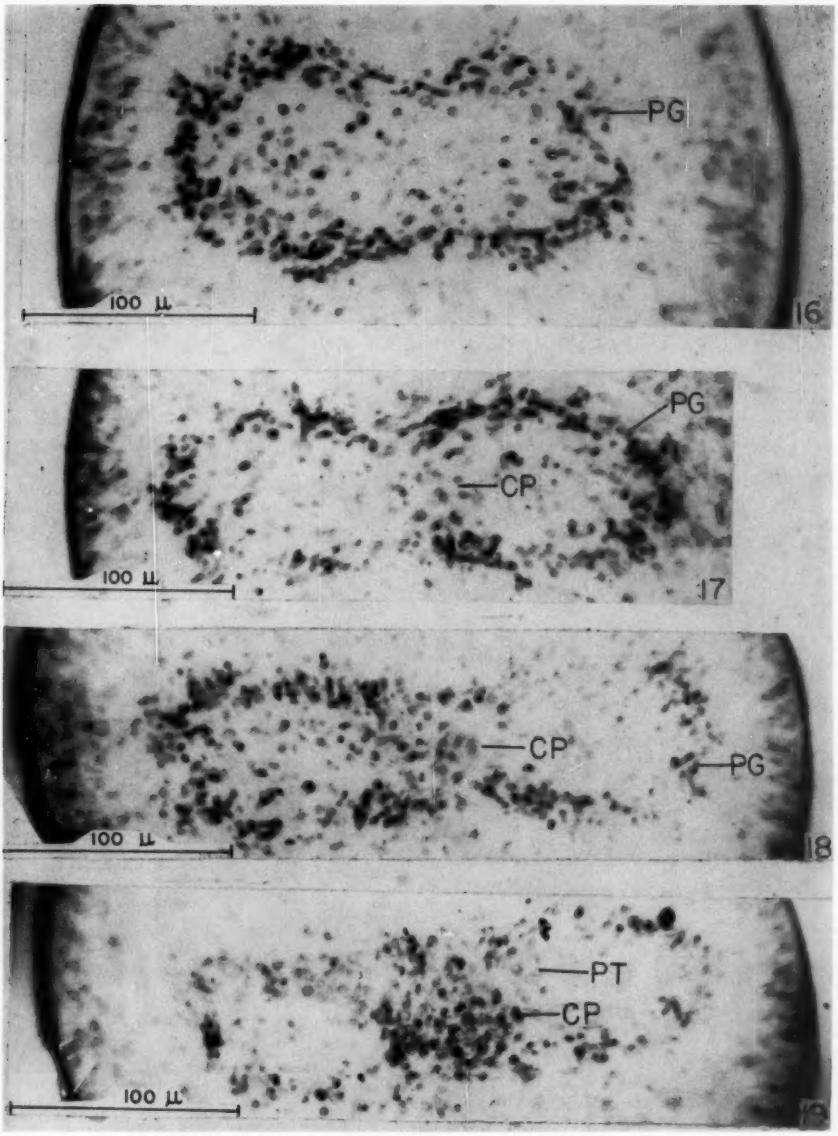
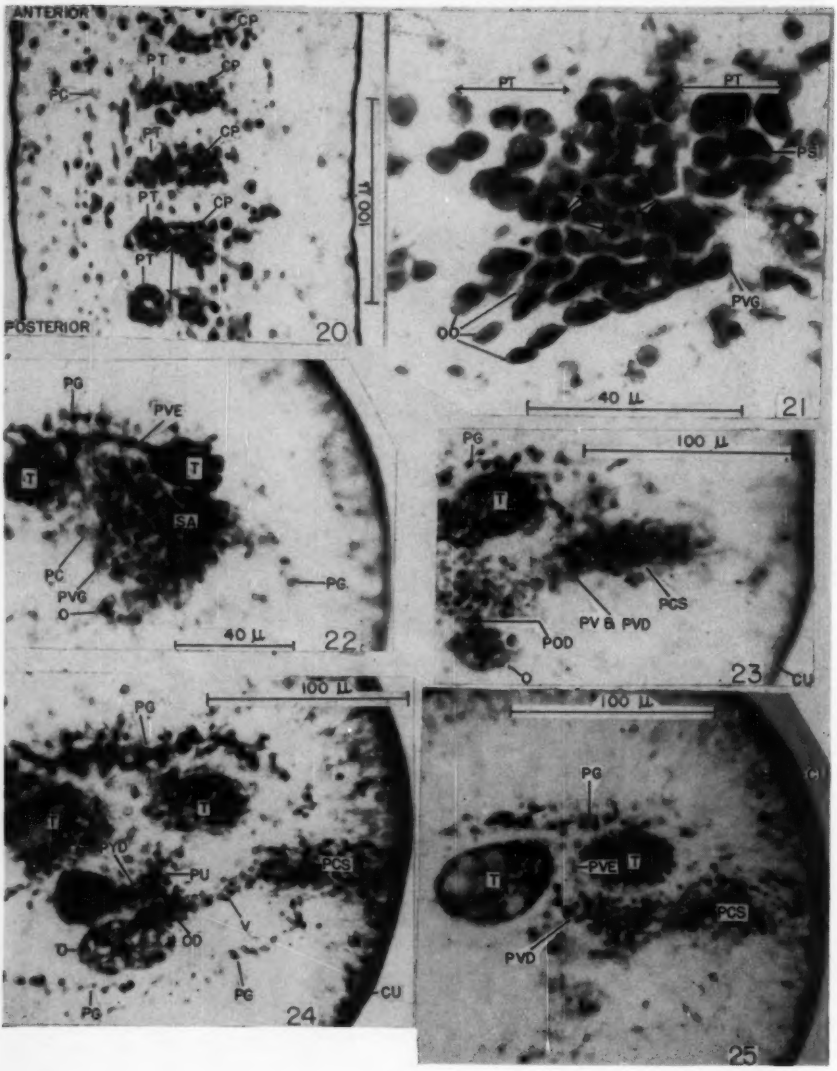


PLATE II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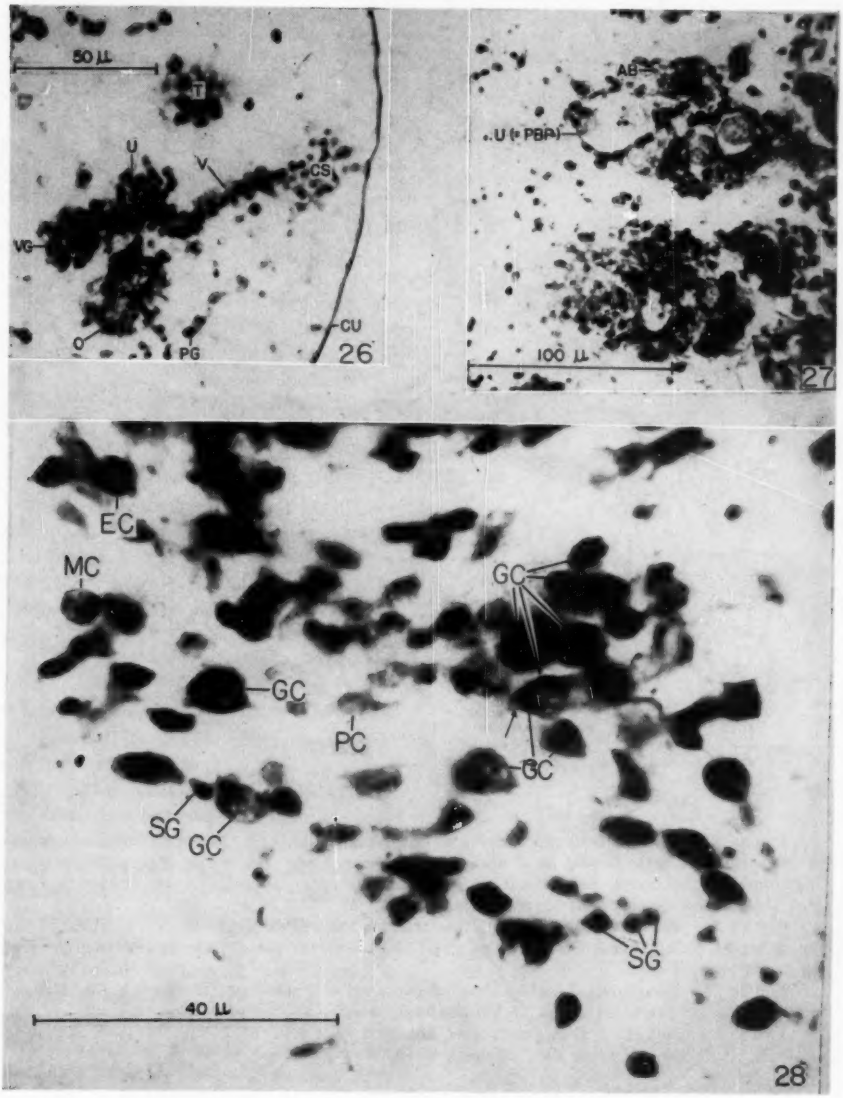
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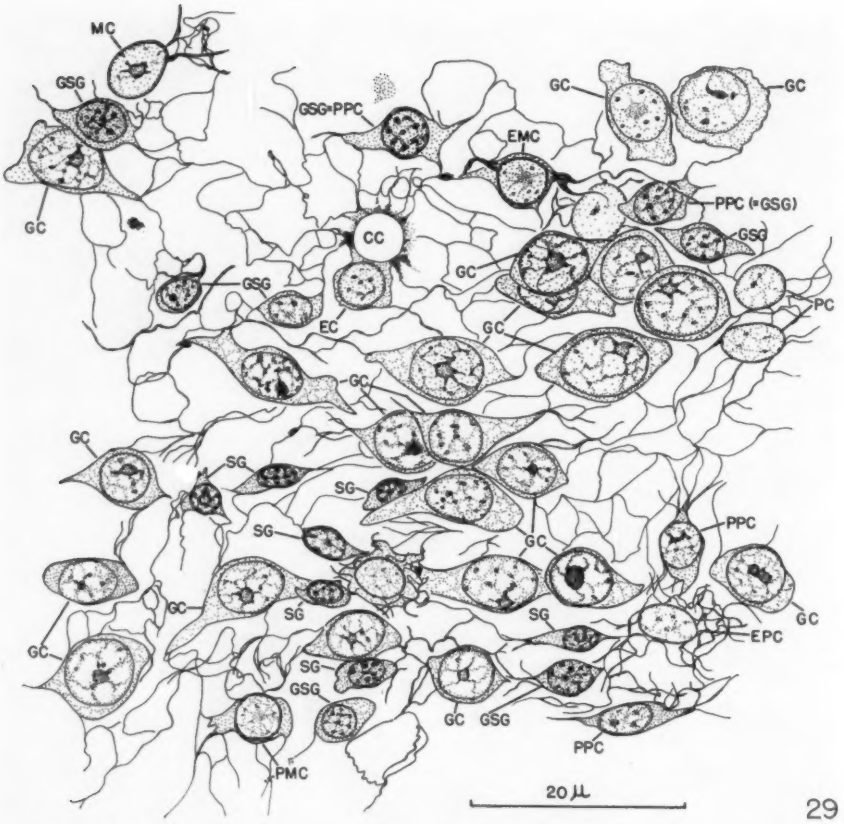

FIGURE 25. Section from the same proglottid as the preceding, showing the male reproductive ducts. In both 24 and 25 a noticeable thickening appears in the peripheral germinal ring dorsal to the testes. Evidence is presented in the next paper that these thickenings are important in the development of paruterine organs.

FIGURE 26. Seven to 10 proglottids posterior to the one in figure 25. In addition to the vagina, ovary, yolk gland and part of one testis and the cirrus sac, the uterus is shown developing from the yolk duct.

FIGURE 27. Longitudinal section showing two uteri with presumptive apical bulbs attached to their anterior surfaces; about 25 proglottids posterior to the preceding.

FIGURES 28 and 29. Enlargements of the central anlage at about the level shown in figure 18. Different cell types are visible in these two figures.

THE DEVELOPMENT OF ORGAN SYSTEMS IN NEMATOTAENIID CESTODES. II. THE HISTOGENESIS OF PARUTERINE ORGANS IN *BAERIETTA DIANA*

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As far as the author has been able to determine, the literature does not contain detailed analyses of cell-types involved in paruterine organ development. Gough (1911) concluded that the paruterine organs of *Avitellina centripunctata* developed from the uterine wall, while Jewell (1916) believed that they were derived from adjacent parenchyma cells in *Cylindrotaenia americana*. Most authors are agreed that the paruterine organs (egg capsule) in *Mesocestoides* arise from specialized cells near the posterior end of the uterus (see Hamann, 1885; Hoeppli, 1925; Byrd and Ward, 1943; and Chandler, 1946 and 1947). Byrd and Ward referred to these specialized cells as "paruterine cells." The present paper is concerned with the origin and early morphogenetic movements of cell components in the paruterine organs of *Baerietta diana* (Helfer, 1948). The terminology as well as the technique used here was discussed in the previous paper of the series (Douglas, 1961).

OBSERVATIONS

Paruterine organs of *B. diana* are dorsoventrally paired (fig. 1), each organ being composed of a basal portion (BP) and an apical bulb (AB). After embryos complete the uterine phase of their development, the uterus becomes modified as the basal portions of the paruterine organs (also it contributes to the egg capsules) and the embryos move into the adjacent apical bulbs. The apical bulbs always contain embryos in advanced proglottids.

Since the basal portions of the paruterine organs appear to be a simple elaboration of the uterine wall (see the sequence of figures 8, 9, and 1), their formation is easily observed in whole mounts and will not be considered here in detail (for an account of uterine formation see Douglas, 1958). On the other hand, apical bulb development is almost impossible to trace in whole mounts (or longitulinal sections) be-

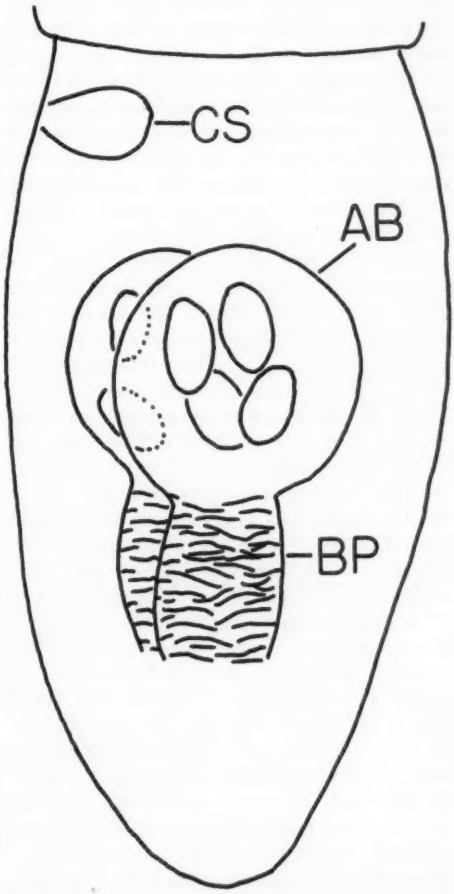

FIGURE 1. The paruterine organs of a terminal proglottid in *B. diana*. They are paired and disposed dorsoventrally to each other; cirrus sac is laterally placed. AB, apical bulb; BP, basal portion of the paruterine organs; CS, cirrus sac × 165.

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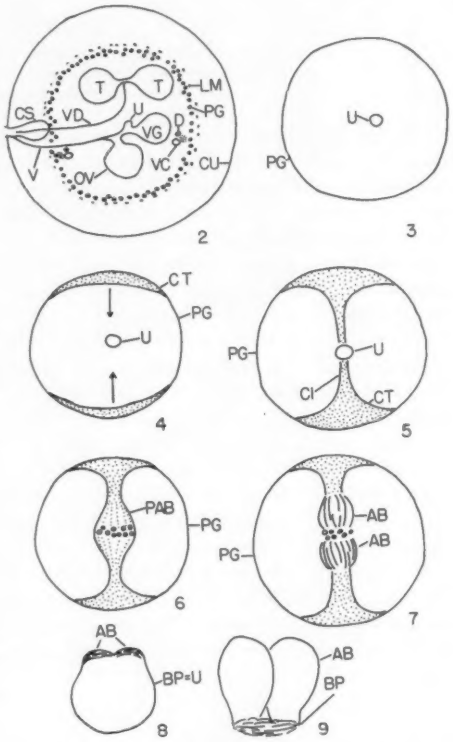
cause the cell masses involved are not brought into geometrical arrangements favorable for study. Therefore, it is necessary to use cross sections where the process is relatively easy to follow. Most of the cell movements involved in formation of apical bulbs occur in the six or seven proglottids immediately posterior to the earliest uterus.

In sections showing the earliest uterus, cell thickenings appear in the dorsal and ventral quadrants of the peripheral germinal ring (CT in figures 2, 4, 10 and 15). These thickenings are accompanied by an increase in mitotic figures whose relative abundance are represented by blackened cells in figures 10 and 11. In 601 nuclei of the dorsal and ventral quandrants, 17 mitotic figures were counted (index 2.8 percent) whereas 874 nuclei in the lateral quadrants of the peripheral germinal ring contained only 10 mitotic figures (index 1.1 percent). The cells in these two thickenings soon begin to migrate centrally toward the anterior surface of the uterus (figures 5, 12, 16 and 17). Migration from the dorsal quadrant nearly always precedes any change in the ventral thickening (compare figures 11 and 12, also see figures 16 and 17). Even though it has not been possible to observe the actual translocation of these cells in living material, the following points of evidence strongly suggest that such cell movements do occur. (1) Soon after the uterus first appears, the zones between the dorsal and ventral quadrants and the uterus are filled with cells resembling those (somatic generative cells) which are located in the peripheral germinal ring itself. (2) In these intermediate zones, the somatic generative cells tend to be spindle-shaped and oriented in a direction which would favor movement, via ameboid action or traction by muscle fibers, toward the center of the proglottid (figure 14). (3) Other than somatic generative cells, the only cell type available in abundance at the time of paruterine organ formation is the parenchyma cell (PC in figure 14). These cells have large spherical (or elongate) nuclei and very little cytoplasm is closely associated with the nucleus. In contrast, somatic generative cells (PGC, fig. 14), are relatively small and their nuclei are enclosed in rounded or fusiform cytoplasm. (4) At this level of development, somatic generative cells occur only in the peripheral germinal ring, the paruterine primordium, and in limited zones between these two cell masses. Parenchyma cells are distributed throughout the proglottid.



FIGURES 2–9. Summary of the early morphogenesis of paruterine organs. 2, a mature proglottid in cross section showing all major structures; 3, same as 2 except that only the peripheral germinal ring and uterus are shown; for explanation see text. 4–9; some of these figures can be compared to plate figures at the end of the paper; those which correspond with each other are: 4 with 10 and 15, 6 with 12 and 17, and 7 with 13. Abbreviations: AB, apical bulb; BP, basal portion of the paruterine organ; CC, central cell mass between presumptive apical bulbs; CS, cirrus sac; CT, cellular thickening in the peripheral germinal ring; DC, dorsal excretory canal; DQ, dorsal quadrant of the peripheral germinal ring; LM, longitudinal muscle fiber; MC, myocyte; OV, ovary; PAB, presumptive apical bulb; PC, parenchymal cell; PG, peripheral germinal ring; PGC, cells of the peripheral germinal ring; PT, cell thickening which is presumed to develop into the apical bulbs; T, testis; U, uterus; V, vagina; VC, ventral excretory canal; VD, vas deferens; VG, vitelline gland; VQ, ventral quadrant of the peripheral germinal ring. × 80.

After most of the somatic generative cells have moved into the region of the apical bulbs, they become fibrous, and their nuclei become arranged in a row or cluster extending from left to right (figures 6, 7, 13 and 14). Fibers elaborated by paruterine cells (transformed somatic generative cells) displace (or perhaps apply themselves to) the anterior wall of the uterus (figure 8) and begin to assume the shape of dorso-ventrally paired bulbs (figures 6, 7, 8, 13 and 18). Evidently subsequent growth of the apical bulbs is accomplished by addition of fibers from paruterine cells until the definitive size is reached.

DISCUSSION

From the observations reported in this paper, it is evident that paruterine organs of *B. diana* are derived from two sources: (1) the uterine wall (developing into the basal portions), and (2) the somatic generative cells of the peripheral germinal ring (which gives rise to the apical bulbs). The participation of the uterus in forming paruterine organs has been reported previously (Ransom, 1911, and Byrd and Ward, 1943) and, therefore, formation of the basal portions of the paruterine organs does not present anything new (actually fibers from the apical bulbs may surround the basal portions later in strobila development, but the present material does not permit this conclusion; perhaps connective tissue stains could reveal whether this process occurs).

However, the retention of primitive cells in posterior proglottids for the genesis of new structures (here apical bulbs) involves, to some extent, a new principle in cestode development. That is, the peripheral germinal ring becomes visible immediately posterior to the scolex and remains encircling the reproductive glands and ducts until its cells move toward the early uterus (see Douglas, 1961). Even so, the reader will think of numerous examples in which adult animals retain relatively undifferentiated cells for the purpose of establishing and maintaining tissues. Consequently, the author submits that introducing this new principle is more in keeping with histogenesis in other animals, than to suppose that previously differentiated cells (viz, parenchyma) would somehow abandon their function to become part of an entirely different tissue.

SUMMARY

Study of serial cross sections of *Baerietta diana* strongly suggests that the apical bulbs of paruterine organs are derived from somatic generative cells in the peripheral germinal ring. These cells appear to migrate centrally from two foci of the peripheral germinal ring (one dorsal and one ventral). Subsequent to migration these somatic cells become fibrous and are arranged in a pattern suggestive of apical bulbs. The basal portion of the paruterine organs seems to be derived from the uterine wall.

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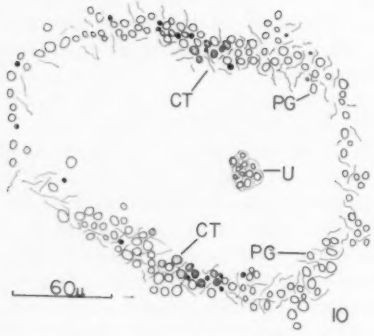
PLATE I



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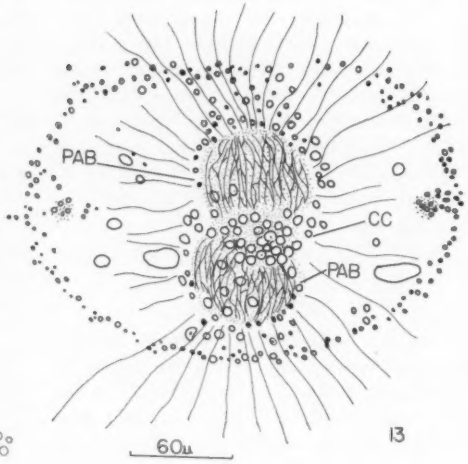

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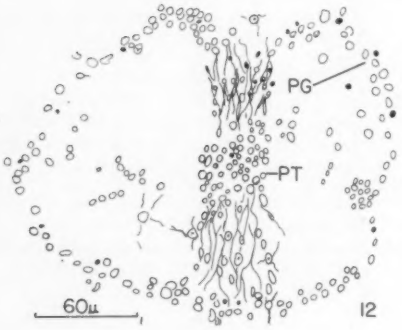

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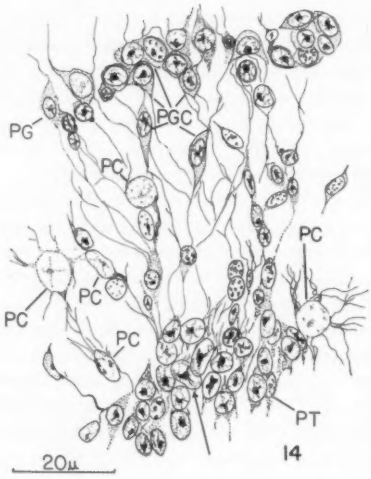

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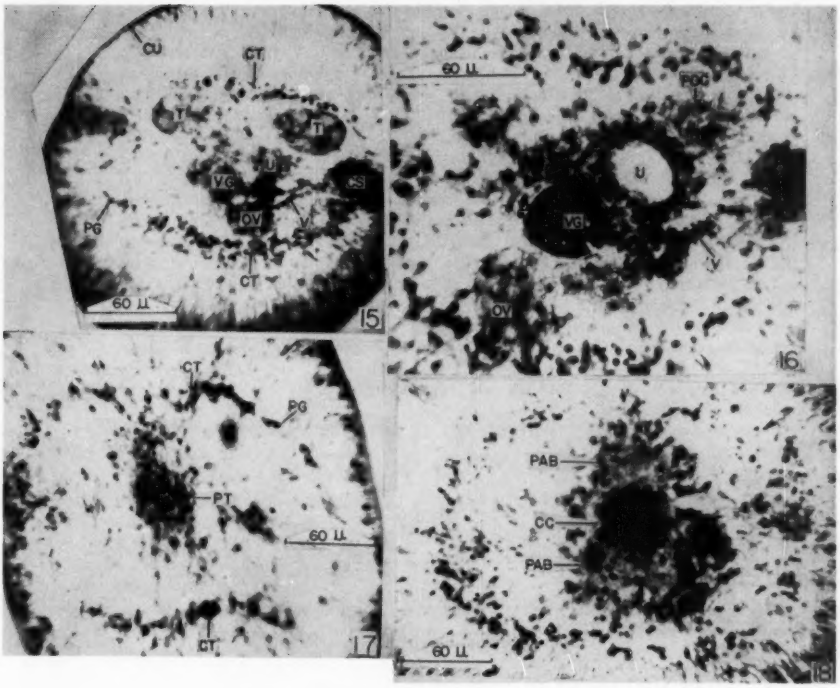
PLATE II



EXPLANATION OF PLATES I AND II

All drawings were made with the aid of a camera lucida from material fixed in Duboscq-Brasil, sectioned at 12 microns and stained with Galigher's haematoxylin and fast green. Photographs were from the same worms. For abbreviations see descriptions of figures 2 to 9.

FIGURES 10 AND 15. Cross section at the level of the early uterus (in figure 10 the centrally located reproductive glands and ducts are omitted). In figure 10 dividing cells are represented as black dots.

FIGURES 11 AND 16. Cross section at a level slightly posterior to the preceding. A zone of cells has developed between the uterus and dorsal quadrant of the peripheral germinal ring.

FIGURES 12 AND 17. Cross section immediately anterior to the uterus in the third proglottid posterior to the one shown in figures 11 and 16. Cell zones have developed both dorsally and ventrally between the uterus and the peripheral germinal ring.

FIGURES 13 AND 18. Later cross section showing the primordia of the apical bulbs.

FIGURE 14. Detailed view of the cells which occur between the peripheral germinal ring and the uterus.

(*Continued from Cover 4*)

THE DEVELOPMENT OF ORGAN SYSTEMS IN NEMATOTAENIID CESTODES. II. THE HISTOGENESIS OF PARUTERINE ORGANS IN *Baerietta diana*. (HELFER, 1948). **Lee T. Douglas** (681–685)

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Emile A. Malek, L. B. Ash, H. F. Lee and M. D. Little (619–623)

THE LIFE CYCLE OF *Plagiorchis goodmani* N. COMB. (TREMATODA: PLAGIORCHIIDAE).
Haig H. Najarian (625–634)

THE IDENTITY OF *Echinoparyphium flexum* (LINTON, 1892) DIETZ 1910 (TREMATODA: ECHINOSTOMATIDAE). Haig H. Najarian (635–636)

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STANDARDIZATION OF TECHNIQUES FOR INFECTING MICE WITH *Toxocara canis* AND OBSERVATIONS ON THE NORMAL MIGRATION ROUTES OF THE LARVAE.
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CANINE MICROFILARIASIS IN EASTERN UNITED STATES.
Nathaniel Rothstein, K. E. Kinnamon, M. L. Brown and E. W. Carithers (661–665)

A FIFTH SPECIES OF *Neoechinorhynchus* (ACANTHOCEPHALA) IN TURTLES.
R. M. Cable and F. M. Fisher, Jr. (666–668)

THE DEVELOPMENT OF ORGAN SYSTEMS IN NEMATOTAENIID CESTODES. I. EARLY HISTOGENESIS AND FORMATION OF REPRODUCTIVE STRUCTURES IN *Baerietta diana* (HELFER, 1948). Lee T. Douglas (669–680)

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Volume 47 August 1961 Number 4—Section 2

Program and Abstracts of the 36th Annual Meeting, American Society of Parasitologists, Lafayette, Indiana August 27–31, 1961

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The Journal of Parasitology

Vol. 47, No. 4 Section 2 August 1961

PROGRAM AND ABSTRACTS OF THE THIRTY-SIXTH ANNUAL MEETING OF THE AMERICAN SOCIETY OF PARASITOLOGISTS

LAFAYETTE, INDIANA

August 27–31, 1961

NOTE: Registration in Memorial Center from Sunday through Wednesday. Obtain Luncheon tickets at registration.

PROGRAM

SUNDAY AFTERNOON, AUGUST 27, 2:00 P.M., ROOM 206, MEMORIAL UNION.

COUNCIL MEETING: OFFICERS AND MEMBERS-AT-LARGE OF COUNCIL, COUNCIL REPRESENTATIVES OF AFFILIATE SOCIETIES, AND COMMITTEE CHAIRMEN. All Past Presidents are cordially invited to attend. (As necessary, an evening session will convene at 7:30 P.M.)

Society Headquarters: Room 206 Memorial Union.

MONDAY MORNING, AUGUST 28.

Session 1. Fowler Hall, Memorial Center

President JUSTIN M. ANDREWS, Presiding.

4. **ZEIN-ELDIN, E. AHMAD and SCOTT, J. ALLEN,** University of Southern California, Los Angeles. The in vitro uptake of Co^{60} vitamin B_{12} by *Ascaris lumbricoides.*

2. **AREAN, VICTOR M., and CRANDALL, CATHERINE A.,** College of Medicine, University of Florida, Gainesville. The effect of immunization on the fate of injected *Ascaris* larvae in the rabbit.

3. **BERGER, H., WOOD, I. B., and WILLEY, C. H.,** American Cyanamid Co., Agricultural Division, New York University, N. Y. Observations on the development and egg production of *Ascaris suum* in rabbits.

4. **ZEIN-ELDEN, E. AHMAD and SCOTT, J. ALLEN,** University of Texas Medical Branch, Galveston. Effects of *Litomosoides carinii* infections on the plasma proteins of cotton rats.

5. **SHARP, A. D. and OLSON, L. J.,** University of Texas Medical Branch, Galveston. Responses of isolated gut segments from nematode-infected guinea

pigs to homologous and heterologous antigens.

6. **ZIMMERMAN, W. J., HUBBARD, E. D., SCHWARTE, L. H., and BIESTER, H. E.,** Veterinary Medical Research Institute, Iowa State University, Ames. Studies on trichiniasis in Iowa wildlife (1953–61).

7. **ROBINSON, EDWIN J., JR.,** Kenyon College, Gambier, Ohio. Survival of *Trichinella* in stressed hosts.

8. **BRIGGS, N. THEODORE,** University of Chicago, Ill. Immunological tissue injury produced by antigens from *Thichinella spiralis.*

9. **KILHAM, L. and OLIVIER, L.,** Division of Biologics Standards and Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Md. The effect of *Trichinella* infection on encephalomyearditis (EMC) virus infections in rats.

10. **LITTLE, M. D.,** Tulane University School of Medicine, New Orleans, La. Concurrent infections of *Strongyloides venezuelensis* and *S. ratti* in wild rats in New Orleans.

11. **KATZ, FRANK F.,** The Jefferson Medical College, Philadelphia, Pa. The *Strongyloides ratti* worm burden in gonadectomized and sham operated male and female rats.

12. **SHIRAKAWA, ALICE M. and KATZ, FRANK F.,** The Jefferson Medical College, Philadelphia, Pa. The enumeration of the free-living stages of *Strongyloides ratti* isolated from coprocultures of gonadectomized and sham operated male and female rats.

Read by title

13. **OLSON, L. J.,** The University of Texas, Galveston. Distribution of *Toxocara canis* larvae in normal mice, and in mice infected with *Toxocara, Ascaris lumbricoides* or *Trichinella spiralis.*

14. **WILLIAMS, JAMES C. and MAYHEW, ROY L.,** Louisiana State University, New Orleans. Preliminary report of ecological studies on larvae of *Cooperia punctata, Oesophagostomum radiatum* and *Trichostrongylus axei.*

15. **LELAND, S. E., JR.,** Department of Animal Pathology, University of Kentucky, Lexington. Some aspects of experimental infection of the Mongolian Gerbil (*Meriones unguiculatus*) with *Trichostrongylus axei.*

MONDAY AFTERNOON, AUGUST 28

Session 2. Fowler Hall, Memorial Center

President-Elect LESLIE A. STAUBER, Presiding

16. **FERGUSON, DONALD L., GOLDSBY, ALICE I., LINDSEY, ARCHIE M.,** Jensen Salsbery Laboratories, Inc., Prairie Village, Kansas. Attempted immunization of dogs against hookworm infection by inoculation of unaltered *Ancylostoma* cell extracts and live-celled tissue sections.

17. **GUEVARA, A., MARTINEZ-TORRES, C., WARREN, L. G., and ROCHE, M.,** Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela. Preliminary study of oxidative metabolism in the dog hookworm.

18. **WOOD, I. B., and PANKAVICH, J. A.,** American Cyanamid Co., Princeton, N. J. The differential susceptibility of the hookworm *Ancylostoma caninum* during its maturation in dogs to the anthelmintic action of disophenol.

19. **HWANG, JOSEPH C.,** Beltsville Parasitological Laboratory, U. S. Department of Agriculture, Beltsville, Md. Cockroaches as carriers of the poultry tapeworm, *Syngamus trachea.*

20. **MAYHEW, ROY L.,** Louisiana State University, New Orleans. Further observations on immunity to *Cooperia punctata, Oesophagostomum radiatum* and *Trichostrongylus axei.*

21. **WILSON, GRANT I.,** Beltsville Parasitological Laboratory, U. S. Department of Agriculture, Beltsville, Md. Serum protein changes in lambs and kids after exposure to the thread lungworm, *Dictyocaulus filaria.*

22. **ENGELBRECHT, HARLEN J.,** Fort Dodge Laboratories, Iowa. An experiment demonstrating the safety and potency of x-irradiated *Dictyocaulus viviparus* larvae vaccine in calves.

23. **LELAND, S. E., JR.,** Department of Animal Pathology, University of Kentucky, Lexington. The in vitro cultivation of the parasitic stages of *Cooperia punctata, Cooperia onchophora, Ostertagia ostertagi,* and *Ostertagia circumcinta:* a preliminary report.

24. **LELAND, S. E., JR., DRUDGE, J. H. and DILLARD, R. P.,** Department of Animal Pathology, University of Kentucky, Lexington. The influence of superimposed nematode infection plus grain supplement on the serum proteins of pastured calves.

25. **KNAPP, S. E. and REED, W. H.,** Oregon State University, Corvallis. Observations on ova production of *Haemonchus contortus* in lambs.

26. **ALLEN, R. W. and SAMSON, K. S.,** Animal Disease and Parasite Research Division, U. S. Department of Agriculture, University Park, New Mexico. Preliminary report on the immunization of sheep with a relatively nonpathogenic strain of *Haemonchus* from pronghorn antelope.

27. **SYMONS, L. E. A. and FAIRBAIRN, D.,** Institute of Parasitology, MacDonald College, McGill University, Quebec, Canada. Digestive function and nippostrongylosis of the rat.

28. **HALEY, A. JAMES,** University of Maryland, College Park. Development of the rat nematode, *Nippostrongylus brasiliensis* (Travassos, 1914) in an abnormal host, the hamster.

TUESDAY MORNING, AUGUST 29.

Session 3. Fowler Hall, Memorial Center.

Council Member CLARK P. READ, Presiding.

29. **WEINSTEIN, PAUL P.,** National Institute of Health, Bethesda, Md. The specific concentration of a reddish pigment in the coelomocytes of some nematodes exposed to vitamin B_{12} in vitro.

30. **WEINSTEIN, PAUL P. and SAWYER, THOMAS K.,** National Institutes of Health, Bethesda, Md. Survival of adults of *Dirofilaria uniformis* in vitro and their production of microfilariae.

31. **SAWYER, THOMAS K. and WEINSTEIN, PAUL P.,** Nation Institutes of Health, Bethesda, Maryland. Survival of *Dirofilaria immitis* microfilariae in modified physiological saline solutions.

32. **PACHECO, GUILLERMO.** Tulane University, New Orleans, La.

Serological studies on dogs experimentally infected with *Dirofilaria immitis.*

33. **ESSLINGER, JACK H.,** Tulane University, New Orleans, La. Vector-parasite relations of *Brugia pahangi:* infection of the mosquito by microfilariae.

34. **TINER, JACK D.,** Rutgers University, New Brunswick, N. J. Laboratory cultures of the plant parasitic nematode genus *Pratylenchus:* a new instrument and a new nematocide test.

Presidential Address. Fowler Hall, Memorial Center

Parasitism and Allergy. **JUSTIN M. ANDREWS,** National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. (Introduced by **GORDON H. BALL,** University of California, Los Angeles.)

Annual Luncheon and Business Meeting. South Ballroom, Memorial Union
President JUSTIN M. ANDREWS, Presiding.

TUESDAY AFTERNOON, AUGUST 29

Discussion Symposium. Fowler Hall, Memorial Center

Parasites of Waterfowl. Presented jointly by the American Society of Parasitologists and the Wildlife Disease Asociation. Organized and presided over by **CARLTON M. HERMAN,** Patuxent Wildlife Research Center, Laurel, Md.

TUESDAY EVENING, AUGUST 29

Session 4. Demonstration; Tour of laboratories of School of Veterinary Medicine; Refreshments. Fowler Hall, Memorial Center

(Laboratories will be open all Tuesday afternoon for arranging demonstrations.)

35. **BULLOCK, W. L.,** University of New Hampshire, Durham. A preliminary study of the histopathology of *Acanthocephala* in the vertebrate intestine.

36. **ROTHMAN, ALVIN H. and ROSARIO, BENJAMIN,** Rice University and Baylor University College of Medicine, Houston, Texas. The structure of the surface of *Macracanthorhynchus hirudinaceous* as seen with the electron microscope.

37. **TINER, JACK D.,** Rutgers University, New Brunswick, N. J. Preliminaries to an increased reliance on automation in biological laboratory research.

38. **CRITES, JOHN L.,** Ohio State University, Columbus. A comparison of some nematodes (Rhigonematidae) parasites of Millipedes.

39. **CROSS, JOHN H. and SCOTT, J. ALLEN,** University of Arkansas Medical Center, Little Rock, and University of Texas Medical Branch, Galveston. A modified method for the culture and isolation of larvae of intestinal nematodes.

40. **ULMER, MARTIN J.,** Iowa State University and Iowa Lakeside Laboratory, Ames. Avian Schistosomes of the genus *Gigantobilharzia* at Lake Okoboji, Iowa.

41. **ROGERS, WALLACE A. and ULMER, MARTIN J.,** Iowa State Uni-

versity and Iowa Lakeside Laboratory, Ames. Effects of selfing on *Hymenolepis nana* (Cestoda: Cyclophyllidea).

42. **CALENTINE, R. L. and ULMER, M. J.,** Iowa State University and Iowa Lakeside Laboratory, Ames. Studies on the *Archigetes-Biacetabulum* relationship.

43. **HERBER, ELMER C. and TREJOS, ALFONSO,** Dickinson College, Carlisle, Pa., and University of El Salvador, San Salvador, C. A. Some trematodes from El Salvador.

44. **WAITZ, J. ALLAN,** University of Illinois, Urbana. Studies on the ultrastructure of larval Hydatigera taeniaeformis.

45. **WOLFGANG, ROBERT W.,** Hess and Clark Research Laboratory, Ashland, Ohio. The efficacy of cadmium compounds in the treatment of *Echinococcus multilocularis* in dogs and rodents.

46. **HARKEMA, REINARD and MILLER, GROVER C.,** North Carolina State College, Raleigh. A new strigeoid trematode (*Diplostomatidae: Alariinae*) from Florida raccoons.

47. **SHORT, ROBERT B.,** Florida State University, Tallahassee. Dicyemid mesozoans from the coast of Florida.

48. **FRIED, B. and PENNER, L.,** University of Connecticut, Storrs. Studies on ocular trematodiasis II. Growth of *Philophthalmus* sp. in vivo and on the chorioallantois of chicks.

49. **OGREN, R. E.,** Dickenson College, Carlisle, Pa. Continuity of cells from Onchosphere to early Cysticercoid in the development of *Hymenolepis diminuta* (Cestoda: Cyclophyllidae).

WEDNESDAY MORNING, AUGUST 30

Session 5. (Concurrent with Session 6) Fowler Hall, Memorial Center

Council Member LOUIS J. OLIVER, Presiding.

50. **JENSEN, THORKIL and DUBES, GEORGE R.,** University of Kansas School of Medicine, Lawrence. Cloning, titration, and differentiation of *Acanthamoeba* sp. by plating.

51. **DIAMOND, L. S., MERYMAN, H. T. and KAFIG, E.,** National Institutes of Health, Bethesda, Maryland. Storage of frozen *Entamoeba histolytica* in liquid nitrogen.

52. **GOLDMAN, MORRIS and GLEASON, NEVA N.,** Communicable Disease Center, Chamblee, Georgia. Antigenic relationships of two strains of *E. histolytica* and one of *E. hartmanni* as shown by cross-sorption of fluorescent antisera to each strain.

53. **HOFFMAN, G. L., DUNBAR, C. E., and BRADFORD, A. D.,** U. S. Department of the Interior, Leetown, W. Va. Studies on *Myxosoma cerebralis* (Hofer) Plehn (Protozoa: Myxosporidae) the cause of whirling disease of trout.

54. **WARREN, L. G.,** Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela. Competition between glucose and galactose in the culture form of *Schizotrypanum cruzi*. Preliminary report.

55. **GRUN, JOHN,** Rugers University, New Brunswick, N. J. The stability of a strain of *Leishmania donovani*.

56. **(Withdrawn by authors.)**

57. **DUNAGAN, T. T.,** Purdue University, Lafayette, Indiana. A comparative study of protein fractions of Acanthocephala by the "Fingerprint" technique.

58. **HUGGHINS, ERNEST J. and DAUMAN, CARL F.,** South Dakota State College, Brookings. *Mediorhynchus grandis* (Acanthocephala: Gigantorhynchidae) in a wild turkey of South Dakota.

59. **BULLOCK, WILBUR L.,** University of New Hampshire, Durham. A preliminary study of the histopathology of Acanthocephala in the vertebrate intestine.

60. **PENNER, LAWRENCE R. and FRIED, BERNARD,** University of Connecticut, Storrs. Studies on ocular Trematodiasis. I. Marine acquired Philophthalmiasis.

61. **FRIED, BERNARD and PENNER, LAWRENCE R.,** University of Connecticut, Storrs. Studies on ocular Trematodiasis. II. Growth of *Philophthalmus* sp. in vivo and on the Chorioallantois of chicks.

Read by title

62. **WALTON, ARTHUR C.,** Knox College, Galesburg, Illinois. The protozoa as parasites of Amphibia. I.

63. **WALTON, ARTHUR C.,** Knox College, Galesburg, Illinois. The protozoa as parasites of Amphibia. II.

64. **WALTON, ARTHUR C.,** Knox College, Galesburg, Illinois. The protozoa as parasites of Amphibia. III.

65. **WENRICH, D. H.,** University of Pennsylvania, Philadelphia. Comments on *Balantidium "suis"*.

66. **FERRAL, D. JOANNE, DOUGHERTY, ELLSWORTH C. and HOLINKA, CHRISTIAN F.,** University of California, Berkeley. Tumors in an axenically grown species of microannelid, *Enchytraeus fragmentosus* Bell, 1951.

67. **SINDERMANN, CARL J.,** U. S. Bureau of Commercial Fisheries Biological Laboratory, Boothbay Harbor, Maine. Sporozoan parasites of sea herring.

68. **LOTZE, J. C., LEEK, R. G., SHALKOP, W. T., and BEHIN, R.,** Beltsville Parasitological Laboratory, Md. Coccidial parasites in the "wrong host" animal.

69. **DORAN, D. J. and FARR, M. M.,** Agricultural Research Service, Beltsville, Md. Bacteria-free suspensions of *Eimeria acervulina* sporozoites and the effect of antibiotics on excystation.

WEDNESDAY MORNING, AUGUST 30

Session 6. (Concurrent with Session 5) Room L. A. 34, Lynn Hall

Custodian WILFORD S. BAILEY, Presiding

70. **BONFANTE, RAFAEL G. and FAUST, ERNEST CARROLL,** University of Valle, Cali, Colombia. Parasitologic surveys in Cali, Departamento del Valle, Colombia. X. Enterobiasis in school boys of Bario Siloe, Cali, Colombia.

71. **LEPAK, JOSEPH W. and THATCHER, VERNON E.,** University

of Texas Medical Branch, Galveston. The development of *Nematospiroides dubius* in an abnormal site in the white mouse and some apparent effects on the serum protein of the host.

72. **BURROWS, ROBERT B., HUNT, GEORGE R. and LILLIS WILLIAM G.,** The Wellcome Research Laboratories, Tuckahoe, N. Y. A new series of pyridines with anthelmintic activity.

73. **DRUMMOND, R. O.,** U. S. Department of Agriculture, Kerrville, Texas. A new organophosphorus systemic insecticide for the control of larvae of *Oestrus ovis* L. in sheep.

74. **WOOD, I. B., EMRO, J. E. and WALETZKY, E.,** American Cyanamid Company, Princeton, N. J. The anthelmintic effect of a sulfamoylphenyl phosphorothioate on the gastrointestinal nematodes of ruminants.

75. **CUCKLER, ASHTON C.,** Merck Institute for Thereapeutic Research, Rahway, N. J. Thiabendazole, a new broad spectrum anthelmintic.

76. **CAMPBELL, WILLIAM C.,** Merck Institute for Therapeutic Research, Rahway, N. J. Effect of thiabendazole upon infections of *Trichinella spiralis* in mice, and upon certain other helminthiases.

77. **EGERTON, JOHN R.,** Merck Institute for Therapeutic Research, Rahway, N. J. The effects of thiabendazole upon *Ascaris* and *Stephanurus* infections.

78. **TIMMERMAN, J. A., TURNER, H. F., and ARTHUR, B. W.,** Auburn University, Auburn, Alabama. Anthelmintic activity and metabolism of ruelene administered to sheep.

79. **ALICATA, JOSEPH E.,** University of Hawaii, Honolulu. On the ineffectiveness of thiabendazole against the migrating larval stages of the swine kidney worm (*Stephanurus dentatus*) in rabbits.

80. **DRUDGE, J. H. and ELAM, GEORGE,** University of Kentucky, Lexington. Preliminary observations on the resistance of horse strongyles to phenothiazine.

81. **MARQUARDT, WILLIAM C.,** Montana State College, Bozeman. The action of some phenothiazine derivatives against the free-living stages of *Nematodirus spathiger*.

Read by title

82. **DRUDGE, J. H., WYANT, Z. N., and ELAM, GEORGE,** University of Kentucky, Lexington. Observations on the efficacy of three phenothiazine preparations on a phenothiazine-resistant strain of *Haemonchus contortus*.

83. **DRUDGE, J. H. and ELAM, GEORGE,** University of Kentucky, Lexington. Comparison of thiabendazole, rueline and phenothiazine for anthelmintic activity in sheep.

84. **DRUDGE, J. H., WYANT, Z. N., ELAM, GEORGE and ROTHENBERGER, G.,** University of Kentucky, Lexington. Synergistic action between phenothiazine and piperazine-carbon disulfide complex against horse strongyles.

WEDNESDAY AFTERNOON, AUGUST 30

Session 7. (Concurrent with Session 8) Fowler Hall, Memorial Center

Council Member RALPH V. BANGHAM, Presiding.

85. **BAILEY, W. S., DIAMOND, D. L., and WALKER, D. F.,** Auburn University, Auburn, Alabama. Observations on the use of thiabendazole in sheep and cattle.

86. **CIORDIA, H., and BIZZELL, W. E.,** U. S. Department of Agriculture, Experiment, Georgia. A preliminary report on the effects of *Bacillus thuringiensis* var. *thuringiensis* Berliner on the development of the free-living stages of some cattle nematodes.

87. **HARKEMA, REINARD and MILLER, GROVER C.,** North Carolina State College, Raleigh. Observations on parasitism of the raccoon of Cape Island, South Carolina.

88. **NAJARIAN, HAIG, H.,** University of Texas Medical Branch, Galveston, and University of Michigan, Ann Arbor. Developmental stages in the life cycle of *Plagiorchis goodmani* (Trematoda: Plagiorchiidae).

89. **HALL, JOHN E. and GROVES, ALICE E.,** West Virginia University School of Medicine, Morgantown. Studies on virgulate cercariae from *Nitocris dilatatus* Conrad and their entry into arthropod second intermediate hosts.

90. **CABLE, R. M.,** Purdue University, Lafayette, Ind. and Caraibisch Marien-Biologisch Instituut, Curacao, N.A. A cercaria of the Haploporidae (Trematoda: Digenea) and the affinities of that family.

91. **HERBER, ELMER C. and TREJOS, ALFONSO,** Dickinson College, Carlisle, Pa. and University of El Salvador, San Salvador, C.A. Some trematodes from El Salvador.

92. **SELF, J. TEAGUE, PETERS, LEWIS E. and DAVIS, CLARENCE E.,** University of Oklahoma, Norman. The biology of *Nematobothrium texomensis*, McIntosh and Self, 1955 (Didymozoidae) in the buffalo fishes of Lake Texoma.

93. **ANDERSON, ROY C. anud FYVIE, AUDREY,** Ontario Research Foundation and Ontario Department of Lands and Forests, Canada. Observations on *Aelurostrongylus falciformis* (Schlegel) of *Mustela vison* in Ontario.

94. **VERNBERG, WINONA B.,** Duke University Marine Laboratory, Beaufort, North Carolina. The influence of temperature on metabolic response in larval trematodes.

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Session 8. (Concurrent with Session 7) Room L.A. 34, Lynn Hall

Council Member J. ALLEN SCOTT, Presiding.

95. **SENGBUSCH, HOWARD G.,** State University of New York, Buffalo. Observations on the mechanism of sperm transfer in the Oribatei (Acarina).

96. **FELDMAN-MUHSAM, B., and HAVIVI, Y.,** The Hebrew University, Jerusalem, Israel. On the development and function of new accessory glands of Ixodid ticks connected with oviposition.

97. **SONENSHINE, D. E., CLIFFORD, C. M. and KOHLS, G. M.,** University of Akron, Ohio, and Rocky Mountain Laboratory, Hamilton, Montana. The identification of larvae of the genus *Argas* (Acarina: Argasidae).

98. **STABLER, ROBERT M. and BERTHRONG, MORGAN,** Colorado College and Penrose Hospital, Colorado Springs. *Wohlfahrtia opaca* myiasis in an adult human being.

99. **KRISHNAMURTI, B. L., PEARDON, D. L., TODD, A. C., and Mc-**

GIBBON, W. H., University of Wisconsin, Madison. A blood parasite from chickens in Wisconsin.

100. **EDGAR, S. A. and SEIBOLD, C. T.,** Auburn University, Auburn, Alabama. A new species of chicken coccidium.

101. **REID, W. M., SHARMA, N. N. and KEENER, JOYCE,** University of Georgia, Athens. Intestinal species of coccidia in chickens from Georgia.

102. **FARR, M. M. and DORAN, D. J.,** U. S. Department of Agriculture, Beltsville, Md. In vivo excystation of *Eimeria acervulina.*

103. **DORAN, D. J. and FARR, M. M.,** U. S. Department of Agriculture, Beltsville, Md. In vitro excystation of *Eimeria acervulina.*

104. **KANTOR, S. and KENNETT, R.,** American Cyanamid Co., Princeton, N. J. The activity of chlortetracycline against *Eimeria acervulina.*

105. **McLOUGHLIN, D.K . and GARDINER, J. L.,** U. S. Department of Agriculture, Beltsville, Md. Zoalene tolerance by *Eimeria tenella.*

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Session 9. Fowler Hall, Memorial Center

Council Member DALE A. PORTER, Presiding.

106. **LAUTENSCHLAGER, E. W. and CARDELL, R. R., JR.,** University of Virginia and Edsel Ford Institute for Medical Research, Charlottesville, Va. Ultrastructure of the cuticular region and flame-cell system of the Metacercaria, *Diplostomulum trituri.*

107. **ETGES, FRANK J.,** University of Cincinnati, Ohio. Chemotactic responses of snails infected with *Schistosoma mansoni.*

108. **DERY, DONALD W.,** College of Charleston, S. C. Host hibernation and its effects on *Schistosomatium douthitti* and *Schistosoma mansoni* (Trematoda: Schistosomatidae).

109. **STIREWALT, M. A.,** Naval Medical Research Institute, Bethesda, Md. Schistosomule vs. cercaria.

110. **RADKE, MYRON G., RITCHIE, L. S. and ROWAN, WILLIAM B.,** Walter Reed Army Institute of Research, Washington 12, D. C., WHAIR Field Team and U. S. Public Health Service, San Juan, Puerto Rico. Some factors influencing penetration of *Schistosoma mansoni* cercariae in mice exposed to infested flowing waters.

111. **ANDERSON, R. I.,** Walter Reed Army Institute of Research, Washington 12, D. C. Relationship of antibody nitrogen to titer obtained in the cercarial anti slide flocculation test for Schistosomiasis.

112. **LEE, HONG-FANG,** Tulane University, New Orleans, La. Susceptibility of mammalian hosts to experimental infection with *Heterobilharzia americana.*

113. **SADUN, E. H., YAMAKI, A., LIN, S. S., and BURKE, J. C.,** Walter Reed Army Institute of Research, Washington 12, D. C., and 406th Medical General Laboratory, Tokyo, Japan. Resistance induced in mice and in monkeys with the Japanese and Formosan strains of *Schistosoma japonicum.*

114. **MALEK, EMILE A.,** Tulane University, New Orleans, La. Natural and experimental infections of snails and mammals with *Schistosoma bovis.*

115. **VON LICTENBERG, F., SADUN, E. H. and BRUCE, J. I.,** Peter Bent Brigham Hospital, Boston, Mass. and Walter Reed Army Institute of Research, Washington 12, D. C. The histopathology of schistosomiasis in abnormal hosts.

116. **CRANDALL, RICHARD B. and HUNTER, GEORGE W., III.,** University of Florida, Gainesville. Some factors affecting "Immunity" to *Schistosoma mansoni* infections in mice and the possible immunogenic role of the schistosome egg.

117. **RITCHIE, LAWRENCE S., BERRIOS-DURAN, L. A., DEWEESE, R., and ROSA-AMADOR, R.,** U. S. Army Tropical Research Medical Laboratory, Fort Brooke, San Juan, Puerto Rico. Biological potentials of *Australorbis glabratus* (Puerto Rico): Growth rate, age at onset of egg-laying egg production and life span.

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118. **ERICKSON, DUANE G., CALDWELL, DANIEL R., and RITCHIE, LAWRENCE S.,** U. S. Army Tropical Research Medical Laboratory, N. Y. Quantity and infectivity of *Schistosoma mansoni* cercariae emerging from *Australorbis glabratus* maintained on alginated food.

119. **ERICKSON, DUANE G., CALDWELL, DANIEL R., and RITCHIE, LAWRENCE S.,** U. S. Army Tropical Research Medical Laboratory, N. Y. Growth and reproduction of *Australorbis glabratus* fed alginated food.

120. **LEIGH, W. HENRY,** University of Miami, Coral Gables, Fla. Exposure of south Florida snails of the genus *Tropicorbis* to miracidia of *Schistosoma mansoni.*

121. **KOMIYA, YOSHITAKA and KOJIMA, KUNIKO,** National Institutes of Health, Tokyo, Japan. The growth difference of *Oncomelania nosophora* and *O. hupensis,* the vector snails of *Schistosoma japonicum,* between sexes.

122. **YOKOGAWA, M., YOSHIMURA, H., OKURA, T., SANO, M., TSUJI, M., IWASAKI, M. and SHIGEYASU, M.,** The Chiba University, Japan. Follow-up studies of chemotherapy of Paragonimiasis with bithionol.

123. **MIYAZAKI, ICHIRO and KAMO, HAJIME,** Kyushu University Faculty of Medicine and Tottori University School of Medicine, Fukuoka, Japan. The newest lung fluke in Japan, *Paragonimus miyazakii* Kamo, Nishida, Hatsushika et Tomimura, 1961.

124. **SNYDER, RANDALL W., JR. and CHENG, THOMAS C.,** Lafayette College, Easton, Pa. The effect of the larvae of *Glypthelmins pennsylvaniensis* (Trematoda: Brachycoeliidae) on glycogen deposition in the hepatopancreas of *Helisoma trivolvis* (Say).

THURSDAY AFTERNOON, AUGUST 31

Session 10. Fowler Hall, Memorial Center

Editor ELERY R. BECKER, Presiding.

125. **MUELLER, JUSTUS F.,** State University of New York, Syracuse. Experimental inversion of the primary axis in the developing egg of *Spirometra mansonoides.*

126. **ROBERTS, LARRY S.,** The Johns Hopkins University, Baltimore, Md. and MacDonald College, Quebec, Canada. Morphological deelopment and maturation of *Hymenolepis diminuta* in the definitive host as affected by population density.

127. **ROBERTS, LARRY S.,** The Johns Hopkins University, Baltimore, Md., and MacDonald College, Quebec, Canada. The influence of population density on changes in chemical composition of *Hymenolepis diminuta* during its growth in the rat.

128. **COLEMAN, ROBERT M.,** Russell Sage College, Troy, N. Y. In vivo antibody binding sites in *Hymenolepis nana* as demonstrated by direct and indirect immunofluorescent staining.

129. **DOUGLAS, LEE T.,** The Johns Hopkins University, Baltimore, Md. Somatic pairing of chromosomes in normal and mutant strains of *Hymenolepis diminuta.*

130. **VOGE, M. and BERNTZEN, A. K.,** University of California, Los Angeles. In vitro hatching of oncospheres of *Hymenolepis diminuta.*

131. **BERNTZEN, A. K. and VOGE, M.,** University of California, Los Angeles. In vitro hatching of oncospheres of *Hymenolepis citelli* and *Hymenolepis nana.*

132. **HOLMES, JOHN C.,** University of Alberta, Edmonton, Canada. The importance of coyotes (*Canis latrans*) in the maintenance of sylvatic echinococcosis: Preliminary observations.

133. **WEINMANN, C. J. and ROTHMAN, A. H.,** Rice University, Houston, Texas. Effects of natural stresses and of cortisone upon acquired resistance to *Hymenolepis nana* in mice.

134. **OGREN, ROBERT E.,** Dickinson College, Carlisle, Pa. Continuity of cells from oncosphere to early cysticercoid in the development of *Hymenolepis diminuta* (Cestoda: Cyclophyllidea).

135. **STUNKARD, HORACE W.,** The American Museum of Natural History, N. Y. *Cycloskrjabinia taborensis* (Loewen, 1934) and systematics of the cestode family, Anoplocephalidae.

136. **MEYER, MARVIN C. and VIK, ROLF,** University of Maine, Orono. *Sparganum sebago,* incidence and location in host fishes.

137. **FREEMAN, REINO S.,** Ontario Research Foundation, Toronto, Canada. The life history of *Proteocephalus parallacticus* MacLulich, 1943 from lake trout.

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138. **NADAKAL, A. M.,** Immaculate Heart College, Los Angeles, Calif. Structure and development of paruterine organs in *Avitellina centripunctata* (Rivolta, 1874).

139. **NADAKAL, A. M.,** Immaculate Heart College, Los Angeles, Calif. Frequency and seasonal fluctuations of infections of sheep with certain helminth parasites in northern India.

140. **COLEMAN, R. M. and FOTORNY, N.,** Russell Sage College, Troy, N. Y. Antigenic analysis of *Hymenolepis* extract by gel diffusion.

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ABSTRACTS

1. **The In Vitro Uptake of Co^{60} Vitamin B_{12} by** *Ascaris lumbricoides.* S. G. ZAM, L. J. THOMAS, JR. AND W. E. MARTIN, University of Southern California, Los Angeles.

Freshly obtained *Ascaris* were labeled in a nutrient medium (Ellison, 1959) containing Co^{60} vitamin B_{12} through a pH range of 4.4 to 8.4. Concentration of the vitamin by the nematode was noted through the entire pH range. Maximum uptake occurs at pH 6.4, with males absorbing more of the vitamin than the females. Greater external concentration of the vitamin produces increased vitamin absorption by the nematode. On a microgram per g basis, larger females bind less B_{12} than smaller females. No such relationship was observed in the males. Incubation for twelve hours as pH 6.4 with 10^{-3} M 2,4-dinitrophenol or iodoacetic acid added to the medium results in no uptake of the vitamin. Sodium cyanide (10^{-3} M) incubation however does not inhibit the vitamin uptake by the nematode. Distribution after 12-hour incubation in the radioactive vitamin from greatest concentration (microgram per g) to least were as follows: gut, peri-enteric fluid, muscle, reproductive system, and cuticle. Incubation of labeled worms for one week in a medium devoid of vitamin B_{12} is accompanied by a decrease of the labeled vitamin from the gut and peri-enteric fluid and an increase in the reproductive system in both sexes.

2. **The Effect of Immunization on the Fate of Injected** *Ascaris* **Larvae in the Rabbit.** V. M. AREAN AND C. A. CRANDALL, University of Florida, Gainesville.

Infective larvae of *Ascaris suum* were hatched in vitro and injected in the ear vein of rabbits previously immunized by intraperitoneal injections of lyophilized *Ascaris* larvae. Challenged non-immunized rabbits and immunized non-challenged rabbits served as controls. The rabbits were sacrificed at regular intervals of time and the histopathologic changes in the lungs studied.

Within the first 4 hours post injection numerous larvae surrounded by eosinophils were found in the capillaries. This response did not differ in magnitude between immunized and non-immunized rabbits. Thereafter the cellular response was greater in the immunized rabbits. In these, a diffuse interstitial response was noted even in regions where no larvae were seen. Giant cells appeared about degenerating and intact larvae as early as 24 hours post injection; granulomas were well developed by the second day and on the twelfth day only degenerating larvae were detected. Larvae were readily seen in non-immunized rabbits on both the 6th and 12th days and some larger larvae were observed in the alveoli. Serum was taken at regular intervals throughout the experiment and antibody titers determined. A correlation between serologic and histologic findings were made. (This investigation was supported by a research grant (E-3212) from the National Institutes of Health, Public Health Service.)

3. **Observations on the Development and Egg Production of** *Ascaris suum* **in Rabbits.** H. BERGER, I. B. WOOD, AND C. H. WILLEY, Agricultural Division, American Cyanamid Company, Pearl River, New York, and Department of Biology, New York University, New York.

It has been generally believed that the larvae of the swine roundworm *Ascaris suum* fail to complete their development in small laboratory mammals and pass out of these hosts soon after migrating through their lungs. However, the following data show that males and females of *A. suum* continue development in the intestine and can reach maturity in the rabbit.

Male New Zealand White rabbits, 6 to 8 weeks old, were inoculated with an average of 4000 embryonated eggs of *A. suum* from swine. Egg counts and postmortem examinations were used to obtain the data.

In the first test, 3 of 6 rabbits contained mature and immature adults at necropsy, 44 to 47 days postinoculation.

In a second experiment, all of 15 rabbits contained worms in the intestine at necropsy, from 10 to 99 days after inoculation. Generally, more worms were present before 22 days, but 1 rabbit harbored 677 immature adults at 44 days. All 4 rabbits killed during the ninth week were infected with 1 to 6 adults. In a third test only 3 of 19 rabbits were infected at necropsy, between 53 days and 68 days after inoculation.

Typical eggs of *A. suum* appeared in the feces as early as 46 days after inoculation. Data from 8 rabbits indicated that a mature female ascarid produced from 15,000 to 45,000 eggs per day. When cultured in 1 percent formalin at 27 C, the eggs were fully embryonated in 23 days.

The possible effects of these intestinal stages of *A. suum* should now be considered in immunological studies in the rabbit and perhaps in other laboratory hosts.

4. **Effects of *Litomosoides carinii* Infections on the Plasma Proteins of Cotton Rats.** E. AHMED ZEIN-ELDIN AND J. ALLEN SCOTT, University of Texas Medical Branch, Galveston.

Comparative electrophoretic studies on plasma proteins have revealed certain naturally occurring differences between suspectible and resistant hosts of *Litomosoides carinii*. Examination of plasma from 72 uninfected cotton rats, of a strain which is susceptible to infection, showed the following fractions occurring in the given mean percents: gamma globulin, 2.59; fibrinogen, 5.76; beta-one globulin, 9.77; alpha-two globulin, 30.16; and albumin, 50.89. The normal plasma of cotton rats lacks beta-two and alpha-one globulins as compared to naturally resistant rodents, e.g., rice rats and white rats. Seven cotton rats with infections of 6 to 9 months duration show an apparent increase in the gamma and the beta-one globulins, while the alpha-two globulin seems to remain constant. The albumin fraction, on the other hand, seems to suffer a loss of approximately one fifth of its normal value. In two other cotton rats where the infection lasted for the unusually short period of 3 to 4 months the increase in gamma and beta-one globulins occurred at a slightly higher level with apparent further loss of albumin. In another group of six cotton rats with recent infections, it appears that during the early migratory and developmental phase of the infection only the gamma globulin fraction increases while the other fractions remain close to the normal values. This work was supported by National Institutes of Health Grant E-64 and National Science Foundation Grant G-4088.

5. **Responses of Isolated Gut Segments From Nematode Infected Guinea Pigs to Homologous and Heterologous Antigens.** A. D. SHARP AND L. J. OLSON, The University of Texas, Galveston.

Female guinea pigs were orally infected with either *Ascaris lumbricoides, Toxocara canis,* or *Trichinella spiralis,* or were injected subcutaneously with metabolites or sonicate from *Trichinella* larvae. Animals were necropsied 3.5 to 4.5 weeks after infection or injection for Schultz-Dale tests with saline soluble extracts from *Ascaris*-egg or *Toxocara-egg* antigens, or with the *Trichinella* antigens above. Both primary and consecutive challenge of a gut segment with these antigens were done to obtain information on cross-reactions between these three systems. Homologous and heterologous responses were obtained in tests with isolated gut segments, and also with whole animal responses following intracardiac challenge.

6. **Studies on Trichiniasis in Iowa Wildlife (1953–61).** W. J. ZIMMERMANN, E. D. HUBBARD, L. H. SCHWARTE, AND H. E. BIESTER, Veterinary Medical Research Institute, Iowa State University, Ames.

Studies have been made on the sylvatic occurrence of *T. spiralis* in wildlife of Iowa. During the 1953–61 period examinations were made of 7070 wildlife specimens, representing approximately 44 species. Trichinae have been found in fourteen species, namely rat, mink, fox, opossum, raccoon, striped skunk, spotted skunk, coyote, badger, beaver, least weasel, wolverine, fox squirrel, and horned owl. Two or more infected species have been obtained from 11 of 12 primary sampling areas with a maximum of 7 reservoir species from a central Iowa area. (Supported in part by a research grant, E505 (C5), from the National Institute of Health, U. S. Public Health Service.)

7. **Survival of *Trichinella* in Stressed Hosts.** EDWIN J. ROBINSON, JR., Kenyon College, Gambier, Ohio.

Enhanced parasite survival in psychologically stressed hosts is reported. *Trichinella spiralis* and an inbred strain of laboratory mouse were used. Experimental mice were stressed for 2 days prior to infection, in a device which subjected them to intermittent brief periods of a mild electrical stimulation, bright lights, and a loud noise. Experimentals and controls then were given an accurately determined number of trichina larvae via stomach tube, and the experimental group stressed for 6 to 12 more days. In one case, mice immunized by a prior infection were used.

A greater proportion of the worms was recovered from the stressed mice in all cases. If the post-infection stressing was not continued for at least 12 days, the attrition of worms in the control hosts did not proceed far enough to give a statistically significant difference in the mean numbers of worms recovered from the 2 groups. In 3 experiments, with 12 days of post-infection stressing, the difference was significant. No worms were found in the immunized control mice, but a considerable number were alive after 12 days of stressing of the immunized experimental group.

The use of psychologically stressed laboratory mice as a tool in studying immunity is convenient and feasible. The findings of these experiments will be discussed with relation to the physical origin of the native intestinal partial immunity, and acquired immunity.

8. **Immunological Tissue Injury Produced by Antigens from *Trichinella spiralis*.** N. THEODORE BRIGGS, The University of Chicago.

In vitro and in vivo studies have characterized the reactivity of rodent tissues to antigens from *Trichinella*. Following sensitization of mice with somatic antigens suspended in Freund's adjuvant, the presence of hypersensitivity was demonstrated by exposing mast cells from such mice to homologous antigen preparations. The resultant cell damage, as evidenced by metachromasia and cell disruption with release of granules, was similar to that produced by exposure of mast cells from infected mice to either metabolic or somatic antigens. Degranulation was not noted after exposure of mast cells from uninfected mice to the same concentrations of these antigens or after exposure of mast cells from *Trichinella*-infected mice to certain heterologous antigens. This host cell sensitivity appeared approximately 2 weeks after a single infection with 50 larvae and persisted for at least 15 months after primary infection. It has also been shown that mast cells from *Trichinella*-infected rats are similarily sensitive. Such cell injury has been shown to be associated with the release of biologically active amines.

9. **The Effect of *Trichinella* Infection on Encephalomyocarditis (EMC) Virus Infections in Rats.** L. KILHAM, Division of Biologics Standards, and L. OLIVER, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, Maryland.

10. **Concurrent Infections of *Stronglyoides venezuelensis* and *S. ratti* in Wild Rats in New Orleans.** M. D. LITTLE, Tulane University School of Medicine, New Orleans.

Infections of *Strongyloides venezuelensis* Brumpt, 1934, and *S. ratt*i Sandground, 1925, were found together in 6 of 8 wild rats (*Rattus norvegicus*) trapped in New Orleans. The parasitic females of *S. venezuelensis predominated* in each case. The two species differ morphologically the spiralling of the ovaries around the intestine in *S. venezuelensis;* in *S. ratti* the ovaries are straight. This feature was constant in *S. venezuelensis*, being present in each of 175 mature females derived from a pure infection established in the albino rat by exposure to a single infective larva and passsed in rats through 5 generations. The two species differ in the shape of the mouth. Moreover, *S. ratti* discharges hatched and unhatched larvae in the hosts' feces while *S. venezuelensis* discharges only eggs containing cleavage stages. The free living stages, not previously described for *S. venezuelensis*, include those of both homogonic and heterogonic cycles, depending upon conditions within the cultures.

Since strains of *Strongyloides* isolated from rats are commonly used for various types of experiments, the necessity of identifying the species of *Strongyloides* obtained for experimental purposes from wild rats becomes evident in view of these findings.

11. **The *Strongyloides ratti* Worm Burden in Gonadectomized and Sham-Operated Male and Female Rats.** FRANK F. KATZ, The Jefferson Medical College, Philadelphia.

Sexually mature female Wistar rats were spayed or sham operated 10 days prior to receiving subcutaneously approximately 1150 filariform larvae in 0.1 ml tap water. Male rats were castrated 10 days or sham operated 9 days prior to receiving the same inoculum on the same day. The inoculation process was such that no member of a group followed another of the same group.

The rats were starved 24 hours prior to being killed 7 to 14 days post-inoculation. Longitudinally opened segments of the entire small intestine were incubated in saline in conical flasks 4 to 7 hours at 37 C. After 24 to 48 hours refrigeration, the sediment was poured into Petri plates and covered with 20 percent glycerine in 70 percent ethyl alcohol. The alcohol was allowed to evaporate and the worms were counted directly under a stereoscopic microscope.

Fourteen spayed females, 14 control females, 12 castrated males and 14 control males were involved in the analysis of the data. The worm counts ranged from 96 for a control male rat to 794 in the case of a spayed female. The mean number of worms per spayed female, control female, castrated male, and control male was 484, 390, 402, and 393, respectively. The results were examined statistically by an analysis of variance and all effects (gonadectomy, sex, and gonadectomy-sex interaction) were found to be nonsignificant, the probability values being >0.1, >0.2, and >0.2, respectively. (This investigation was supported by a Public Health Service research grant (E-2894) from the National Institute of Allergy and Infectious Diseases).

12. **The Enumeration of the Free-living Stages of *Strongyloides ratti* Isolated from Coprocultures of Gonadectomized and Sham Operated Male and Female Rats.** ALICE M. SHIRAKAWA AND FRANK F. KATZ, The Jefferson Medical College, Philadelphia.

In three consecutive experiments, one on females and two on males, sexually mature Wistar rats were surgically treated prior to receiving subcutaneously approximately 45 filariform larvae in 0.6 ml tap water. Females were spayed or sham-operated 7 to 10 days and males were castrated or sham operated 6 to 12 days prior to being inoculated.

Feces were collected in water in pans from 6 P.M. to 6 A.M. beginning in the evening of the 3d day post-inoculation and ending the morning of the 25th day. Cultures were made each morning, using per animal a maximum of 10 g feces to 20 g bone charcoal in one Petri dish, and incubated 48 hours at 26 C. The worms were isolated in a Baermann apparatus and counted in the tapped sample by a dilution technic or after sedimentation.

By concentrating on the counts obtained for the 6th to 15th day post-inoculation, which appeared to be the most informative period, a total of 6 experimental and 9 control female and 15 experimental and 15 control male rats were involved in the analysis of the data. There was no significant difference in the total number of adult worms (males and females combined) recovered from each group. The ratio of filariform larvae to adult worms, however, was significantly higher for female rats than for male rats (probability of $F < 0.001$) while the effect of surgical treatment was of questionable significance (probability of $F < 0.05$). Since the first of the three experiments was concerned with female rats, the results may be a reflection of a shift toward heterogeneity in the nematode. (This investigation was supported by a Public Health Service research grant (E-2894) from the National Institute of Allergy and Infectious Diseases).

13. **Distribution of *Toxocara canis* Larvae in Normal Mice, and in Mice Infected with *Toxocara*, *Ascaris lumbricoides* or *Trichinella spiralis*.** L. J. OLSON, The University of Texas, Galveston.

Serial necropsies of Fairfield-Webster mice infected with *Toxocara* larvae were done to determine by pepsin digestion the organ distribution of larvae, and the gross pathology of the host at different times after primary infection. Larvae were found to follow a definite migration pattern in the various organs of the mouse culminating in a marked invasion of skeletal muscle and central nervous tissues. Prior infection of mice with *Ascaris* or *Toxocara* resulted in a decrease in the total numbers of challenging *Toxocara* larvae reaching extraintestinal sites, and in an alteration of the distribution pattern of these larvae in the organs of the mouse. Prior infection with *Trichinella* did not appear to alter the usual migration pattern or survival of challenging *Toxocara* larvae. In a preliminary experiment dithiazanine (1 mg per kg) given daily for 5 days to mice infected with *Toxocara* did not alter the migration pattern or survival of these organisms as compared to control infections.

14. **Preliminary Report of Ecological Studies on Larvae of *Cooperia punctata*, *Oesophagostomum radiatum* and *Trichostrongylus axei*.** JAMES C. WILLIAMS AND ROY L. MAYHEW, Louisiana State University, New Orleans.

Manure less than 18 hours old from calves with pure infections has been placed in the center of 4-foot square plots surrounded by galvanized iron frames under field conditions. The frames are 11 inches high and dug into the ground to a depth of 5 to 6 inches. They extend above the surface of the ground 5 to 6 inches so that all rain water is held in this 4 ft-square area, thus insuring the possibility of recovery of all larvae hatched from the eggs placed on the soil. Rainfall, soil and air temperature, and humidity records are kept at the experimental area. These studies have been carried on over a period of 2.5 years.

Number of plots on which larvae survived by months.

No. of months		1	2	3	4	5	6	7	8	No. plots that produced no larvae
O. radiatum	grass	11	5	1	1	1	0	0	0	13
	manure	3	6	6	3	4	0	0	0	13
T. axei	grass	8	10	10	1	3	2	0	0	1
	manure	3	6	10	6	5	1	0	0	2
C. punctata	grass	7	12	6	1	1	0	1	1	6
	manure	6	10	7	7	5	7	0	0	5

Moisture on the grass following a rain under field conditions increases the number of

larvae on the grass that are available to the calf. In greenhouse studies 4 times as many larvae were recovered from grass under high-humidity as under dry conditions.

15. **Some Aspects of Experimental Infection of the Mongolian Gerbil** (*Meriones unguiculatus*) **with** *Trichostrongylus axei*. S. E. LELAND, JR., Department of Animal Pathology, University of Kentucky, Lexington.

The Mongolian gerbil was susceptible to experimental infection with *Trichostrongylus axei* from four sources of isolation (two equine and two bovine). Larval doses of 2,000, 4,000, and 6,000 were fatal in three animals. Thereafter, doses of larvae ranged from 250 to 1,500 at increments of 250 in 27 animals. With the latter range, three gerbils succumbed following administration of 1,000 larvae and one following 1,250 larvae. Of 29 gerbils which survived beyond the 20th day post inoculation, 28 became patent as indicated by detection of worm eggs within the feces. During a 61-day post-inoculation period, worm-egg counts at 1- to 3-day intervals ranged from 30 to 19,604 per 24 hours.

Gross pathological manifestations in severe and fatal cases closely resembled those seen in ruminants and the horse. Larvae cultivated from gerbils infected with the two equine isolates of the worm produced patent infections in five gerbils.

Seven gerbils were inoculated with *Haemonchus placei* larvae with doses ranging from 250 to 1,750 at intervales of 250. Patency of *H. placei* was not established in these animals, though subsequent inoculation with bovine *T. axei* produced patent infections.

The technical advantages of this domestic animal pathogen when studied in this small host are numerous. For example, a complete 24-hour fecal sample can be collected and diluted in a 125 ml. erlenmeyer flask and the complete gastrointestinal tract can be digested in 100 ml of artificial digestive juice, thus facilitating worm recovery and counting. Since patent infections can be established with relatively small numbers of infective larvae, this host-parasite system should be useful, for example, in testing infectivity of larvae grown axenically. (A project of the Kentucky Agricultural Experiment Station, published with approval of the Director, and supported in part by National Science Foundation Research Grant G-14215 and contributing to Regional Project S-21, Gastrointestinal Parasites of Ruminants.)

16. **Attempted Immunization of Dogs Against Hookworm Infection by Inoculation of Unaltered Ancylostoma Cell Extracts and Live-Celled Tissue Sections.** DONALD L. FERGUSON, ALICE I. GOLDSBY, ARCHIE M. LINDSEY, Jensen-Salsbery Laboratories, Inc., Kansas.

Certain proteins, protein extracts, and protein fractions have been shown to elicit antibody formation by injection into the host. In this trial unaltered *Ancylostoma* cell extracts and live-celled tissue sections of *Ancylostoma spp* were utilized in an attempt to immunize susceptible dogs against *Ancylostoma* infection. A comparison is made between challenged dogs which received an inoculation of *Ancylostoma* protein and challenged dogs receiving no inoculation. The comparison is made on the basis of nematode numbers, nematode size, dog hemoglobins and serum precipitation (gel-diffusion technique).

17. **Preliminary Study of Oxidative Metabolism in the Dog Hookworm.** A. GUEVARA, C. MARTÍNEZ-TORRES, L. G. WARREN AND M. ROCHE, Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela.

Preliminary studies on the dog hookworm indicate that these parasites are able to utilize relatively high quantities of oxygen and metabolize ingested glucose. Manometric determinations of oxygen consumption in Ringer's solution indicate that female and male worms consume respectively 1600 and 2200 microliters of oxygen per hour per gram wet weight in the absence of added substrate. Endogenous respiration was not stimulated by glucose. Maltose was tested for its effect upon the respiration of females, and the latter sugar did not alter the endogenous rate. The respiratory quotients for females and males were 0.49 and 0.56, respectively.

Worms were studied in the apparatus of Roche and Martínez-Torres (Exp. Parasit. 9: (250, 1960) using dog serum which contained radioactive glucose-C14 or Chromium-Cr51. Experiments with Cr51 indicated that measurable quantities of glucose passed through the digestive tract of the worms during the test periods. Nonetheless, it was not possible to demonstrate reducing substances in the chamber where the digestive products of the worm accumulated. However, it was possible to demonstrate pyruvic acid in excess of that predicted by transport of serum through the worm.

The utilization of glucose and production of pyruvic acid has also been observed in worms maintained for several days in serum with added antibiotic.

18. **The Differential Susceptibility of the Hookworm, *Ancylostoma caninum*, during its Maturation in Dogs to the Anthelmintic Action of Disophenol.** I. B. WOOD, J. A. PANKAVICH, Agricultural Division, American Cyanamid Company, Princeton, New Jersey.

The new antihookworm agent 2,6-diiodo-4-nitrophenol, known generically as disophenol (The trademark of the American Cyanamid Company for disophenol is D.N.P.), was found to be highly effective against adult hookworms 28 or more days after inoculation at 7.5 mg per kg subcutaneously. However, it was inactive at the same dose against 7-day-old infections of hookworms in dogs.

Further critical tests have demonstrated that experimentally infected dogs did not void hookworms if treatment was given prior to 14 days post-inoculation. Small numbers of worms were eliminated on the 14h day, at the same time eggs first appeared in the feces, and there were variable results from 15 to 20 days. Complete effectiveness was achieved against 21-day or older infections.

The effects of disophenol and other types of antihookworm compounds against immature stages will be compared.

19. **Cockroaches as Carriers of the Poultry Gapeworm, *Syngamus trachea*.** J. C. HWANG, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

Three species of cockroaches, *Periplaneta americana*, *Supella supellectilium*, and *Blatella germanica*, after 2 to 3 days' starvation, were exposed to embryonated eggs from 10 macerated female *Syngamus trachea*. Nine 6-week-old turkey poults were separated into 3 equal groups so that a single species of cockroach could be fed to each group. Six cockroaches that had been exposed to gapeworm eggs 24 hours previously were then administered to each bird. In a similar experiment, six specimens per bird of the first two species of cockroaches mentioned that had been exposed to gape worm eggs 48 hours previously were fed to 2 groups of 10 birds of comparable age, respectively. At necropsy 5 weeks after exposure, 2 pairs of gapeworms were recovered from one bird fed *P. americana* and one pair from a bird fed *S. supellectilium* 24 hours after exposure. One pair was also recovered from a bird fed *P. americana* and from two birds fed *S. supellectilium* 48 hours after exposure. Birds fed these two cockroaches 10 days after exposure to embryonated gapeworm eggs and those fed exposed *B. germanica* failed to become infected.

A large number of embryonated eggs and a few active larvae were seen in the crop and intestine of specimens of *P. americana* dissected 24 hours after exposure to eggs of *S. trachea*, and one active gapeworm larva was observed in this cockroach 9 days after exposure. So far as the writer is aware, this is the first report of cockroaches serving as carriers of *S. trachea*.

20. **Further Observations on Immunity to *Cooperia punctata*, *Oesophagostomum radiatum* and *Trichostrongylus axei*.** ROY L. MAYHEW, Louisiana State University, Baton Rouge, Louisiana.

Immunity to *Cooperia punctata* has been demonstrated by reinoculation to persist for 2 years in three animals and in one for 3 years. Three animals that became immune to *Cooperia punctata* have been successfully infected with *Oesophagostomum radiatum*. After becoming resistant to the latter species they were successfully infected with *Trichostrongylus axei*. Two of these then became immune to *Trichostrongylus axei*.

21. **Serum Protein Changes in Lambs and Kids after Exposure to the Thread Lungworm, *Dictyocaulus filaria*.** GRANT I. WILSON, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

Data were obtained at weekly intervals on the changes in the serum proteins of 12 lambs and 12 kids, of which 8 lambs and 8 kids were initially infected when they were about 4 months old with 2,500 or 5,000 infective larvae of *Dictyocaulus filaria*. Ten of these animals were also challenged with 5,000 larvae 9 weeks after the initial infection. Three lambs and 3 kids died from initial infections and 1 kid succumbed to the challenge infection. The surviving animals, including 4 unnfected and 4 challenged controls, were necropsed 19 weeks after the beginning of the experiment.

While the worms were maturing the percentage of gamma globulin increased and the albumin-globulin (A/G) ratio decreased in animals in which the initial infections became well established. These changes were approximately proportional to the number of worms present. The serum proteins returned to within the normal range about 8 weeks after larval production ceased.

The most significant finding was the failure of either initial or challenge infections to change the percentages of the various serum protein fractions of animals that appeared to be naturally resistant to lungworm infection. Challenge exposures did not elicit any changes in the serum proteins of previously infected animals except in the one kid that died 6 weeks after receiving the challenge infection. In this animal, the initial dose of 2,500 larvae was followed by an increase in gamma globulin to 41 percent, but this response did not protect it from the challenge dose. At the time of death, the A/G ratio of the kid was 0.61 and the gamma globulin was 42.4 percent, whereas these values in the control animals were 1.23 and 22.4 percent, respectively.

22. **An Experiment Demonstrating the Safety and Potency of X-Irradiated *Dictyocaulus viviparus* Larvae Vaccine in Calves.** HARLEN J. ENGELBRECHT, Fort Dodge Laboratories, Kansas.

Calves 6 to 8 weeks old were treated as follows: (1) Ten received 1,000 irradiated larvae, 40,000 r, standardized conditions, (s.c), were killed at intervals ranging from 42 hours to 29 days after vaccination. No gross lesions attributal to the vaccination were noted. One larvae was found in one calf. (2) Five were given 1,000 non-irradiated larvae and were killed 42 hours, 90 hours and 29 days after being dosed. No larvae were found at 42 and 90 hours; but 71, 379, and 134 lungworms were found in those killed at 29 days. (3) Seven were given two doses of 1,000 irradiated larvae (40,000 r) (s.c.) with an interval of 30 days between doses. They were killed at intervals ranging from 2 to 29 days after the second dose. No gross lesions attributal to the vaccination were noted; lungworm was found in one calf. (4) Three calves received two doses of 1,000 non-irradiated lungworm larvae with 30 days between doses. One died 20 days after the second dose; the other two were killed on the 29th day after the second dose. Severe pneumonia was found in all three, and no lungworms were found. (5) Five calves received two doses of 1,000 irradiated larvae (40,000 r) (s.c.) and were challenged with 4,000 non-irradiated larvae. Very few gross lesions were noted; an average of 3.8 lungworms per calf were found in the lungs 31 days post-challenge. (6) Three untreated controls were challenged, and extensive pneumonic lesions were noted 31 days post-challenge. An average of 636 lungworms per calf was found in the controls.

23. **The In Vitro Cultivation of the Parasitic Stages of *Cooperia punctata, Cooperia onchophora, Ostertagia ostertagi* and *Ostertagia circumcincta:* A Preliminary Report.** S. E. LELAND, JR., Department of Animal Pathology, University of Kentucky, Lexington.

Utilizing a medium composed of chicken embryo extract, serum, sodium caseinate, vitamins, a hog-liver extract, a balanced salt solution and antibiotics, it was possible in a roller drum to cultivate *Cooperia punctata* to the egg-bearing adult, and *C. onchophora, Ostertagia ostertagi* and *O. circumcincta* to the adult stage from mixed cultures of 3rd-stage infective larvae. Tests to detect the presence of bacteria in the cultures were negative. Fifth stage *C. punctata* were observed within 14 days and egg-bearing females in less than 36 days. Hundreds of infertile eggs have been recovered from the 2-ml cultures. *Haemonchus sp.* and *Oesophagostomum sp.* developed to late 4th stage. *Cooperia punctata* as a monoinoculant has also been cultivated axenically to the 5th stage within 13 days with infertile eggs being observed in the medium within 32 days. Extensive efforts with a variety of media and conditions have been largely unsuccessful with several different isolates of *Trichostrongylus axei* as a monoinoculant. (A project of the Kentucky Agricultural Experiment Station, published with approval of the Director, and supported in part by National Science Foundation Research Grant G-14215).

24. **The Influence of Superimposed Nematode Infection Plus Grain Supplement on the Serum Poteins of Pastured Calves.** S. E. LELAND, JR., J. H. DRUDGE, AND R. P. DILLARD, Department of Animal Pathology, University of Kentucky, Lexington.

Twelve Jersey and Holstein-Fresian calves (7 to 10 months old) from the Station herd were divided into 2 paired groups. Initially, the calves possessed mixed "light" nematode infections. During the grazing period (April to November) each group was maintained separately, on similar paired pastures with both groups being rotated to new pastures following forage depletion.

Group I calves received a pelleted 16 percent crude protein cattle supplement. In addition, at 2 to 4-week intervals, 24,000 to 331,500 mixed infective trichostrongyle larvae were placed on the pellet supplement.

Group II received no supplement or infective larvae.

No clinical sign of parasitism was evident in either group during the study.

Electrophoretic serum fractionation, total serum protein, and hematocrit determinations were made at 2-week intervals for a 7-month period.

That the grain supplement was successful in completely counter-balancing the influence of the superimposed infections was evident from the relative differences in the blood changes between the two groups. In comparison to group II, group I showed a more consistent and prolonged reduction in hematocrit. Hypoproteinemia was temporarily evident in all animals of group I, while normal values were observed in group II. The serum components of Group I calves also showed more extensive quantitative alterations which consisted of a marked increase in alpha-2-globulin which later decreased. As the alpha-2-globulin decreased, the gamma globulin increased markedly.

A reduction in serum albumin also accompanied the globulin changes. (A project of the Kentucky Agricultural Experiment Station, published with approval of the Director, and contributing to Regional Project S-21, Gastrointestinal Parasites of Ruminants.)

25. **Observations on Ova Production of *Haemonchus contortus* in Lambs.** S. E. KNAPP AND W. H. REED, Department of Veterinary Medicine, Oregon State University, Corvallis, Oregon.

An attempt was made to concentrate helminth ova from an experimentally infected lamb by changing from ad lib. feeding to a limited ration consisting of 1 pound of alfalfa hay and 0.25 pound of grain. Previously, the animal had been given 15,000 third-stage *Haemonchus contortus* larvae. A week prior to feed reduction, total ova per day ranged between 4.5×10^6 and 6×10^6. Forty-eight hours after food intake had been reduced, a 50 percent reduction in feces and ova was recorded. Daily ova production for the next 8 days were as follows: 3×10^6, 2.5×10^6, $7.7. \times 10^5$, 6.2×10^5, 3.4×10^4, 1.2×10^4, 0 and 0. The animal was returned to an ad lib. feeding program but no ova were observed in the feces for six subsequent weekly examinations.

A second lamb infected with 10,000 *Haemonchus* ova was placed in a metabolism stall and hourly fecal samples were recovered with the aid of an automatic collection device. Fecal samples (wet weight) were evaluated as to quantity passed per hour and per day. Likewise, ova per g of feces per hour and per day were recorded. Two 96-hour collections (A and B) spaced 2 weeks apart and one 10-day collection (C) were evaluated. In A, fecal content diminished daily as did total ova. In B, fecal content and total daily ova passed were relatively constant.

Data from C provided further support for a correlation between feces and ova. Feed consumption was directly related to these factors. During this trial daily passage of ova varied from 6×10^6 to 11.5×10^6. Evidence of an hourly variation in ova production was obtained.

26. **Preliminary Report on the Immunization of Sheep with a Relatively Non-Pathogenic Strain of *Haemonchus* from Pronghorn Antelope.** REX W. ALLEN AND K. S. SAMSON, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, University Park, New Mexico.

After observing that a strain of *Haemonchus* from pronghorn antelope was relatively non-pathogenic to domestic sheep (Allen, Samson and Schad, 1959, J. Parasit. 45 (Supp.): 47) we explored the possibility of using this strain to immunize domestic lambs against a domestic-sheep strain. Eighteen worm-free lambs were divided into three groups of six each when they were about 12 weeks old. Lambs of group 1 received 10,000 infective larvae of a strain of *Haemonchus* that had been isolated from a non-captive pronghorn in February 1958 and passed through domestic lambs 11 times. Group 2 received 10,000 larvae of a strain that had been isolated from domestic sheep some 6 years previously. Both strains had been maintained as pure infections under controlled conditions. Group 3 served as uninfected controls.

At 63 days post-inoculation, 4 lambs in each of groups 1 and 2 were challenged with 15,000 larvae of the domestic sheep strain; 4 lambs of group 3 received the same inoculum at this time. The intensity of the resulting infections was measured by weight changes, feed consumption, hemoglobin levels, packed cell volume, and worm eggs per g. As compared with the controls (grop 3), lambs which had been inoculated initially with the antelope strain (group 1) proved to be significantly resistant to challenge with the domestic sheep strain. This resistance was not as marked as it was in group 2 where the challenge involved the homologous strain.

(This work was carried out in cooperation with the New Mexico Agricultural Experiment Station.)

27. **Digestive Function and Nippostrongylosis of the Rat.** L. E. A. SYMONS AND D. FAIRBAIRN, Institute of Parasitology, Macdonald College, McGill University, Quebec, Canada.

Infection of the rat by the nematode *Nippostrongylus muris* (Yokogawa, 1920) produces

profound morphological and physiological disturbances of the jejunum which may be fatal. (Symons, L. E. A., Austral. J. Biol. Sci. **13**: 171, 180 and 578, 1960). Protein labelled with I^{131} was less efficiently digested in infected rats. The pancreatic enzymes, trpysin and amylase, were present in considerably lower concentrations in the jejunal contents. When, however, allowance was made for the larger volume of fluid in the jejuna of infected animals, the total amount of either enzyme was found to be about equal to that in normal animals. There was no evidence that the lower activity of trypsin in infected rats was due to inhibition. The enteric enzymes leucine amino-peptidase and maltase, which occurred at very low concentrations in the jejunal contents, were less abundant in the infected mucosa. A large increase in the weight of the jejunal mucosa of infected animals probably represents a true hyperplasia as DNA increased proportionately. On the other hand, the concentration of RNA in these animals increased by 30 percent.

28. **Development of the Rat Nematode, *Nippostrongylus brasiliensis* (Travassos, 1914) in an Abnormal Host, the Hamster.** A. J. HALEY, University of Maryland, College Park.

Haley (1958, Am. J. Hyg. **67**: 331–349) reported that passage of the rat nematode, *Nippostrongylus brasiliensis*, in an abnormal host, the hamster, substantially increased its infectivity for the hamster. This work has been confirmed and extended. New findings include the following: (1) A strain of *N. brasiliensis* that has been maintained in hamsters for 2.5 years is about 13 times as infective for hamsters as the parent rat strain of the parasite. (2) This hamster strain is still highly infective to rats. (3) Repassage of the hamster strain in rats for 4 months has not altered its high infectivity for hamsters. (4) The lungs constitute a major barrier to the development of rat strain *N. brasiliensis* in hamsters, but not to the development of hamster strain in hamsters. (5) Female hamsters are more resistant than males to infection with rat strain, but this difference is greatly reduced in animals infected with the hamster strain of the parasite. (6) Preliminary work has shown that the increased development of *N. brasiliensis* in hamsters is evident after just one or two passages of the parasite in hamsters. The increased development at first is evident only in made hamsters. Increased development of the parasite in female hamsters occurs later.

29. **The Specific Concentration of a Reddish Pigment in the Coelomocytes of Some Nematodes Exposed to Vitamin B_{12} In Vitro.** PAUL P. WEINSTEIN, National Institutes of Health, Bethesda, Maryland.

When vitamin B_{12} was added to axenic cultures initiated with third-stage larvae of *Nippostrongylus muris* or *Ancylostoma caninum*, a reddish pigment accumulated in the vacuoles of the coelomocytes; it was not detected in any other cells of the nematodes. The coelomocytes of *N. muris* remained pigmented as the organisms differentiated to adult worms. Pigment accumulation was visually detectable when B_{12} was added within a range of 0.05 to 100 micrograms per ml medium, but was absent when B_{12} was not added. The media have been either completely synthetic, or contained embryo homogenate and serum. The intensity of pigment accumulation has been directly related to the concentration of B_{12} in the medium. Vital dyes such as phenol red or neutral red gave negative reactions.

N. muris grown from the egg to the third stage in axenic culture in the presence of B_{12} exhibited similar pigment accumulation in the coelomocytes. Occasionally, third stage larvae isolated from charcoal-feces cultures have shown a light accumulation of similar reddish pigment in the coelomocytes. This phenomenon probably represents variability in the B_{12} content of the charcoal cultures due to differences in microbial activity.

The nature of the pigment accumulating in the coelomocytes is not known. It may represent a specific concentration of B_{12}, itself a red pigment, or the formation of some other pigment in response to this vitamin. Preliminary studies with *Strongyloides ratti* and *S. stercoralis* have not shown an accumulation of pigment in coelomocytes of these nematodes exposed to B_{12}.

30. **Survival of Adults of *Dirofilaria uniformis* In Vitro and Their Production of Microfilariae.** PAUL P. WEINSTEIN AND THOMAS K. SAWYER, U. S. Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

D. uniformis adults were maintained in tissue culture medium NCTC 109 with and without rabbit serum in an atmosphere of 5 percent CO_2 in air. The medium was changed daily to remove acidic metabolic products and to determine microfilarial production. Cultures were established with various numbers and combinations of male and female worms isolated from a cottontail rabbit. In medium 109 alone, the filariids lived 10 days. In medium 109 supplemented with serum (5, 10, or 20 percent) most adults survived 3 weeks, but almost all were

dead by the end of the fourth week. The different serum concentrations tested were approximately equally effective in prolonging survival.

Peak microfilarial production occurred during days 2 and 3, then gradually declined, and ceased completely by days 13 to 16. The average numbers of microfilariae produced by different combinations of male and female worms during their period of survival in medium 109 plus 10 percent serum did not differ markedly. For isolated females, the mean number of microfilariae produced per worm was 17,000; for isolated sexual pairs the mean was 20,800; and for groups of 7 females and 3 males, the mean was 16,700 per female. However, significantly lower production of microfilariae per female was obtained in medium 109 alone or medium 109 supplemented with 5 percent serum, as compared to serum supplements at higher concentrations.

Worms derived from a Dutch rabbit and cultured in medium 109 supplemented with a low concentration of calf serum in addition to the rabbit serum showed survival patterns for the adult worms similar to those stated above, but microfilarial production was considerably lower.

31. **Survival of *Dirofilaria immitis* Microfilariae in Modified Physiological Saline Solutions.** THOMAS K. SAWYER AND PAUL P. WEINSTEIN, U. S. Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

The survival of microfilariae freshly removed from blood of infected dogs was studied under a variety of experimental conditions. Preliminary studies were directed towards determining survival time in solutions of various inorganic ions with and without added dextrose.

Inorganic components of Earle's, Gey's, Krebs-Ringer-Phosphate, and Locke's solutions were found to permit survival of *Mf immitis* for 4 to 8 hrs at 37 C and 6 to 13 hrs. at 27 C. Survival time was increased to 10 to 21 hrs. at 37 C and 20 to 45 hrs. at 27 C when these solutions were modified to contain a uniform concentration of phosphate and dextrose plus varied concentrations of bicarbonate. Locke's saline was modified further by omitting, or varying, the molar strength of sodium, potassium, calcium, phosphate, and bicarbonate, and by adding magnesium and dextrose. Increased survival times (4 to 35 hrs at 37 C, and 5 to 55 hrs at 27 C) were obtained with different combinations and concentrations of these components.

In limited studies with NCTC 109 with 3 percent calf serum microfilariae survived 4 days at 27 C and 29 days at 37 C if gassed with 5 percent CO_2 in air. Krebs-Ringer-Phosphate with 0.25 percent dextrose and 1.5 percent sodium caseinate, buffered at pH 7.2 with Tris buffer, permitted survival for 5 days at 27 C but only 40 hours at 37 C. Microfilariae survived in alkaline saline-sugar solutions, but required more neutral solutions for maximal survival in "nutrient" solutions.

32. **Serological Studies on Dogs Experimentally Infected with *Dirofilaria immitis*.** GUILLERMO PACHECO, Tulane University School of Medicine, New Orleans.

Uncertainty in the interpretation of results of serological tests for the filariases possibly is due to undetermined variation in antibody responses that occur at different stages of the infections. The objective of the present study was to follow at short intervals in dogs the antibody response to experimental *Dirofilaria immitis* infection by means of an indirect tanned-cell hemagglutination (IHA) test and a complement-fixation (CF) test. A phosphate buffered saline (0.15 M, pH 7.2) extract of adult male and female *D. immitis* (D-PBS) and an acid soluble fraction (D-M) prepared according to Melcher's (1943) technique were used in the IHA test and a cholesterolized ethanol extract (Danaraj et al, 1959) of adult male and female *D. immitis* was used in the CF test.

The results of the above tests reflected marked changes in host-parasite relationships during the developmental and early reproductive period of *D. immitis* infection. In the CF test, antibodies were first detected when the immature worms started migrating to the heart during the 3d month of infection; then titers increased rapidly and remained at a plateau (1:320) until microfilariae appeared in the blood, then decreased gradually to barely detectable levels at the 9th month of infection. Similar results were observed in the IHA test with D-PBS and D-M, except that antibody titers were noted as early as 3 weeks after infection and titer maxima were much greater, 1:5120 with D-PBS and 1:20,000 with D-M.

33. **Vector-parasite Relations of *Brugia pahangi*: Infection of the Mosquito by Microfilariae.** J. H. ESSLINGER, Tulane University School of Medicine, New Orleans.

Details of the microfilarial behavior of *B. pahangi* in *Anopheles quadrimaculatus* have been studied in dissected and in serially sectioned mosquitoes.

Within 5 minutes after the mosquito has completed feeding a large proportion of the microfilariae have emerged from the mass of agglutinated blood cells and come to lie in the

plasma-filled space adjacent to the midgut wall. By 6 minutes microfilariae begin to penetrate the wall of the midgut, primarily the posterior half. Within 15 minutes nearly all the microfilariae have left the gut and are in the fat bodies or hemocele, especially in the posterior region of the abdomen. The migratory route to the thoracic muscles is principally through the subcuticular fat. Microfilariae enter the flight muscles as early as 30 minutes.

The beginning of pigmental encapsulation of microfilariae was observed as early as 15 minutes after the ingestion of blood.

34. **Laboratory Cultures of the Plant Parasitic Nematode Genus *Pratylenchus*; A New Instrument and a New Nematocide Test.** JACK D. TINER, Rutgers The State University, New Brunswick, New Jersey.

Meadow nematodes (*Pratylenchus penetrans*) that emerge from infection-damaged corn roots sink into collection points of the cold-trap. Each unit in this instrument is a column of sterile liquid nutrient medium (Exp. Parasit. 9: 121) in which submerged roots are held in a 24 C chamber above a 5 C collection point. About 20 infection-darkened roots are placed in the chamber, and after a week the organisms are aspirated with a syringe. The following day the roots are flooded with new medium. Enough nematodes (now 20,000 per unit and week) are produced so that at the rate of 400 per newly excised root, hundreds of subcultures can be initiated.

Roots are excised from kernels treated with Arasan and Semesan Jr. (DuPont Co. dusts) and cultured on 10 ml of plated agar in 2-oz bottles. Surviving seed-borne microbes become apparent soon enough that the contaminated roots are noticed and discarded before the onset of nematode emergence. Inside a glove box, 20 roots are placed in each trap unit. Subsequent to adoption of the present routine, no units have been contaminated. Even so, the nematode suspensions are treated 1 hour with 0.2 mg per ml mercurochrome and rinsed in water before subcultures are infected. Nematodes treated 24 hours are motile, but do not reproduce.

Comparison of nematode numbers in treated (1 mg per agar plate) and control roots allowed detection of nematocidal chemicals, including 1,2-dibromo-3-chloropropane without recourse to greenhouse facilities.

This investigation was supported by research grant E-1552 from the National Institute of Allergy and Infectious Diseases, U.S.P.H.S.)

35. **A Preliminary Study of the Histopathology of Acanthocephala in the Vertebrate Intestine.** W. L. BULLOCK, University of New Hampshire, Durham, N. H. (See Abstract 58)

36. **The Structure of the Surface of *Macracanthorhynchus hirudinaceus*, as Seen with the Electron Microscope.** ALVIN H. ROTHMAN, Rice University, and BENJAMIN ROSARIO, Baylor University College of Medicine; Houston, Texas.

The periphery of this worm has at least two distinct types of structure. The first has a spongy appearance, with the cavities increasing in size medially. The second has the appearance of a palisade, made up of osmophilic and non-osmophilic regions.

Below this most peripheral layer one can distinguish three or four other structural layers which are not distinctly demarcated. They are recognized by their peculiar morphology.

No nuclei, mitochondria, or cell boundaries have been found.

This investigation was supported, in part, by a fellowship, E-6393, from the National Institute of Allergy and Infectious Diseases of the United States Public Health Service.

37. **Preliminaries to an Increased Reliance on Automation in Biological Laboratory Research.** JACK D. TINER, Rutgers The State University, New Brunswick, New Jersey.

The glassware of chemistry and microbiology must be modified if it is to be used to advantage in the study of living organisms such as pure cultures of invertebrates. The large sizes and high degrees of organization of these animals are responsible for their relatively high sedimentation rates and susceptibility to turbulence and vibrations in the medium. The special mechanical setups that are needed to position and synchronize various axenic cultures will serve to focus new instrumentation on biological materials.

Nematode cold-trap units (see abstract 34) evolved through several models and over a few years. They became functional only because it was possible to construct needed accessories. One experiment with the suspended organisms involved infection of a series of roots in IAVG agar. If these were transferred to IAVG medium with or without agar after 15 and 25 days, a 2-month yield of over 13,000 nematodes per g of roots resulted. If solid medium lacked glucose, yields were not more than 6,000 per g. In liquid medium without glucose the yield was below 3,000 per g.

Additional apparatus for the study of parasitic nematodes is now required. This should be designed so that it will fit into a compact and automatic sequence. Perfusion studies appear to offer the best means to shorten the 2- or 3-month incubation period now required for growth and toxicologic studies with *Pratylenchus penetrans*.

Compactly enclosed culture devices could be generally useful for studies that require that the operator be protected, or that unusual physical conditions be maintained. This investigation was supported by research grant E-1552 from the National Institute of Allergy and Infectious Diseases, U. S. P. H. S.

38. **A Comparison of Some Nematode (Rhigonematidae) Parasites of Millipedes.** JOHN L. CRITES, Ohio State University, Columbus.

During a survey of parasites of Ohio arthropods, nematodes were recovered from the intestines and hind gut of millipedes of the family Polydesmidae. These nematodes differ from *Rhigonema infectum* (Leidy, 1849) and *Rhigonemia nigella* Thomas, 1930 which were recovered from millipedes of the family Julidae in the United States. A study of whole mounts, serial sections and en face preparations indicate that a new species is represented. Nematodes from julid millipedes will be compared with those from polydesmid millipedes by demonstration.

39. **A Modified Method for the Culture and Isolation of Larvae of Intestinal Nematodes.** JOHN H. CROSS AND J. ALLEN SCOTT, University of Arkansas Medical Center, Little Rock, and the University of Texas Medical Branch, Galveston.

During our studies on the mouse nematode *Nematospiroides dubius* a method has been employed whereby large numbers of infective stage larvae have been recovered from mouse feces free of fecal debris. This method is a modification of that described by White (1927, Science **66**: 302).

Feces were collected on moist paper toweling and four or five of the moist fecal pellets were placed in each of six small Syracuse dishes (Bureau of Plant Industry Model, 20 mm inside diameter, Arthur H. Thomas No. 9850). The dishes were then placed in a standard size Petri dish containing approximately 5 mm of water. The cultures were then allowed to incubate at room temperature. Within 4 to 5 days the larvae of *N. dubius* hatched from the eggs and began to migrate from the feces, over the edge of the Syracuse dishes, and into the water. After 10 days most of the larvae migrated into the water and they were easily recovered with a pipette. Care was taken not to allow the feces to become too wet. If excess water collected in the Syracuse dishes the larvae would not develop. The yield from these cultures compares favorably with that from previously described methods.

In addition to *N. dubius* this method has been employed to recover larvae of *Ancylostoma caninum* from dog feces, *Trichostrongylus axei* from rabbit feces and *Strongyloides sp.* from monkey feces. (Supported by a National Science Foundation Grant No. G-4088).

40. **Avian Schistosomes of the Genus *Gigantobilharzia* at Lake Okoboji, Iowa.** MARTIN J. ULMER, Iowa State University and Iowa Lakeside Laboratory, Ames.

Ring-billed gulls (*Larus delawarensis*) collected in the vicinity of the Iowa Lakeside Laboratory harbor avian schistosomes belonging to the genera *Gigantobilharzia* and *Ornithobilharzia*. Of 20 ring-billed gulls examined since 1958, five have yielded specimens of avian schistosomes belonging to these genera. At least 2 species of *Gigantobilharzia* are represented in the collection, including *Gigantobilharzia lawayi* Brackett, 1942. A second as yet unidentified species has been found but once in the gastrosplenic vein of a gull examined in 1959. This unusually large male schistosome measuring 83 mm in length is quite similar to a European species (*G. acotylea*) described by Odhner in 1910 from *Larus fuscus* in Sweden, but differs from the latter in lacking well-defined caudal lobes. Despite assiduous search, no additional specimens have been found. (This study was supported in part by a research grant (E-3463) from the National Institute of Allergy and Infectious Disease of the National Institutes of Health.)

41. **Effects of Selfing on *Hymenolepis nana* (Cestoda: Cyclophyllidea).** WALLACE A. ROGERS AND MARTIN J. ULMER, Iowa State University and Iowa Lakeside Laboratory.

Successive generations of selfed *H. nana* have been studied. The indirect cycle of development using *Tribolium confusum* as intermediate host was employed in these experiments. A single cysticercoid was fed to each young, uninfected experimental mouse. The posterior 8 to 10 proglottids of the adult worm, removed from the mouse intestine 11 days post-feeding, were

then fed to beetles. Maintenance of beetles at 30 to 32 C made possible the harvesting of fully developed cysticercoids 6 days after exposure of beetles to eggs. Control mice with multiple infections were also maintained and periodically examined.

Preliminary experiments indicate that number of cysticercoids per beetle appears to decrease markedly after the first selfing of the adult tapeworm. After 4 selfed generations, cysticercoid numbers are reduced approximately by half. The percentage of cysticercoids maturing to adults also decreases after selfing. No adult tapeworms in these preliminary investigations have been recovered after five selfed generations.

Preliminary studies of morphological variations (abnormal number and arrangement of testes, incomplete segmentation, sterility, reversal of genital atrium, fusion of cirri, etc.) indicate that the incidence of such variations does not appear to change significantly as a result of selfing. (This study was supported in part by a grant (G-9022) from the National Science Foundation.)

42. **Studies on the *Archigetes-Biacetabulum* Relationship.** ROBERT L. CALENTINE AND MARTIN J. ULMER, Iowa State University and Iowa Lakeside Laboratory.

Gravid caryophyllaeid cestodes are frequently found in tubificid oligochaetes (*Limnodrilus hoffmeisteri*) in the Iowa River near Iowa Falls, Iowa. As many as three well-developed mature cestodes have been recovered from the seminal vesicle of a single host. Eggs generally are shed by the worms, accumulate within the tubicifid's seminal vesicle, but in some instances are retained in utero. A conspicuous enlargement and expansion of the uterus is associated with the latter condition. In the laboratory, development of eggs taken from the uterus of gravid worms has been studied to the fully formed oncosphere.

Gravid caryophyllaeids, morphologically indistinguishable from those within oligochaetes (save for the lack of a cercomer), have been found in naturally-infected carp (*Cyprinus carpio*). Infections of fish in nature appear to be seasonal, since carp examined from areas of known oligochaete infection were found to harbor large numbers of this parasite in May 1961 but not in the months July to October, 1960.

Careful study of whole mounts and sections indicates that specimens from oligochaetes are similar to those referred to the genus *Archigetes*, while adults from carp are typical of the genus *Biacetabulum*. Life cycle studies now in progress should aid in elucidating the relationships between these two genera. (This study was supported in part by a grant (G-9022) from the National Science Foundation.)

43. **Some Trematodes from El Salvador.** E. C. HERBER, AND A. TREJAS, Dickinson College, Carlisle, Pa. and the University of El Salvador, San Salvador, C.A. (See abstract 91)

44. **Studies of the Ultrastructure of Larval *Hydatigera taeniaeformis*.** J. A. WAITZ, Univ. of Illinois.

Larval *Hydatigera taeniaeformis* were taken from the livers of artificially infected laboratory rats. Pieces of the worms as well as sections of the cyst wall were fixed in a modification of Palade's buffered osmium at pH's 7.4 and 8.4, and were embedded in butyl-methyl methacrylate. After sectioning on a Porter-Blume ultramicrotome the sections were examined with an RCA EMU-II electron microscope.

The cyst wall is composed of mammalian connective tissue which appears to be vascularized mostly by venules. The cuticula of the worm is highly complex in structure and is provided with microvilli on its outer surface. In the lower portions of the cuticle are many small mitochondria. Below the cuticula is a sub-cuticula provided with distinct channels which connect the parenchyma with the cuticula. Cross sections through flame cells show that the flagellae are packed in hexagonal rods and are made up of the typical 9 plus 2 pattern. (This work was supported by a fellowship (EF-9587-C1) from the U. S. Public Health Service.)

45. **The Efficacy of Cadmium Compounds in the Treatment of *Echinococcus multilocularis* in Dogs and Rodents.** ROBERT W. WOLFGANG, Hess and Clark Research Laboratory, Ashland, Ohio.

Two dogs, identical in size and 13 pounds each, were infected experimentally with *Echinococcus multilocularis*. Seven days after infection, one dog was treated with cadmium orthotoluene sulfonate at the rate of 0.025 percent of the feed for 21 consecutive days. One week after the cessation of treatment the dogs were necropsied and the intestines were examined for evidence of tapeworms. The medicated dog showed no infection, while the non-medicated dog showed an overwhelming infection with *E. multilocularis*. The control dog lost 2 pounds; the medicated dog gained 2 pounds in the 5 weeks of the test. The results of this experiment

as well as tests with other cadmium derivatives in dogs and cotton rats will be demonstrated.

46. **A New Strigeoid Trematode** (*Diplostomatidae:Alariinae*) **from Florida Raccoons.** REINARD HARKEMA AND GROVER C. MILLER, North Carolina State College, Raleigh.

Examination of 19 raccoons from Glades County, Florida, in September 1960 revealed the presence of an unknown strigeoid trematode. Five of the 19 raccoons had light to moderate infections of this parasite in the small intestine. These specimens are sufficiently different from any known form to constitute a new genus. They are characterized by an elongated bi-lobed holdfast organ which extends to or beyond the oral sucker. There is similarity to the genus *Pharyngostomoides* Harkema, 1942, in the parallel position of the testes and in general body form. The major features which separate this strigeoid trematode are the position and structure of the holdfast organ, the forward location of the testes, the large recess in the genital atrium, and the much larger size.

47. **Dicyemid Mesozoans from the Coast of Florida.** R. B. SHORT, Florida State University, Tallahassee, Fla. (No abstract)

48. **Studies on Ocular Trematodiasis II. Growth of** *Philophthalmus* **sp. In Vivo on the Chorioallantois of Chicks.** B. FREID AND L. PENNER, University of Connecticut, Storrs. (See abstract 61)

49. **Continuity of Cells from Onchosphere to Early Cysticercoid in the Development of** *Hymenolepis diminuta* **(Cestoda: Cyclophyllidea).** R. E. OGREN, Dickinson College, Carlisle, Pa. (See abstract 134)

50. **Cloning, Titration, and Differentiation of** *Acanthamoeba sp.* **by Plating.** T. JENSEN AND G. R. DUBES, University of Kansas School of Medicine, Lawrence.

Discrete colonies of *Acanthamoeba sp.* were formed when amoebae and the yeast *Candida albicans* suspended together in a nutritional medium containing 0.9 percent agar were plated between 2.2 percent agar layers and incubated 2 to 6 days at 37 C. Colonies were produced by both trophozoites and cysts of *Acanthamoebae sp.* strains V, R, and S derived from three occurrences of amoeba-contaminated vertebrate tissue cultures. Efficiency of plating (eop) of amoebae was high enough and extent of aggregation of inoculated amoebae low enough so that a large fraction, often 0.90 to 0.99, of the colonies produced must have been initiated by individual amoebae, and thus were clones. When trophozoites were inoculated, the number of colonies produced was a linear function of the number of potential colony-forming units (pcfu's) (i.e., individual amoebae plus aggregates) inoculated. When unwashed cysts were inoculated, the function was linear for strain V but for strains R and S larger numbers of pcfu's gave lower eop's. Washed R and S cysts gave linear function when colonies were counted soon after appearance; sometimes at later counts a nonlinear function as above was obtained. The addition of culture medium in which V, R, or S cysts had been produced strongly inhibited colony production by R or S cysts but not by trophozoites or V cysts, thus suggesting that these media contained an inhibitor(s) of excystment for strains R and S. By harvesting organisms from atypical colonies a strain S mutant producing faint colonies and strain V and R mutants producing colonies clearer than usual were obtained.

51. **Storage of Frozen** *Entamoeba histolytica* **in Liquid Nitrogen.** L. S. DIAMOND, Laboratory of Parasitic Diseases, National Institutes of Health, Public Health Service; H. T. MEREYMAN AND E. KAFIG, Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland.

Trophozoites of *Entamoeba histolytica* have been stored in liquid nitrogen following subjection to a process of slow freezing. Amoebae grown in association with a *Crithidia* were suspended in 5 percent dimethylsulfoxide. After an equilibration period of 30 min at 35 C, 290,000 to 780,000 amoebae, contained in 0.2 ml portions, were placed in 1-dram, screw-capped vials. The vials were then placed in a Canalco Slow-Freeze unit maintained at an initial temperature of 0 C and cooled to −35 C at a rate of 1 degree per minute. At this point, they were either (a) transferred to a dry-ice cabinet (−79 C) for periods varying from 1 to 96 hrs, then placed in liquid nitrogen, or (b) transferred directly to liquid nitrogen. Vials were thawed at 45 C by momentary immersion in a water-bath containing a few drops of 25 percent O. T. Aerosol. The criterion of successful preservation was based on ability of the amoebae to survive and reproduce in culture. To insure growth, it was necessary to add additional viable *Crithidia*

to the amoebae suspension either just prior to freezing or immediately following thawing.

Each of 106 vials thawed after storage in liquid nitrogen from 1 to 91 days, has yielded viable amoebae upon culture. No apparent differences in yields of amoebae have been observed between vials stored in liquid nitrogen following initial cooling to −35 C and those subjected to transient storage in dry-ice prior to storage in liquid nitrogen. Attempts to preserve amoebae by rapid freezing in liquid nitrogen without prior slow-freezing have, so far, failed.

52. **Antigenic Relationships of Two Strains of *E. histolytica* and One of *E. hartmanni* as Shown by Cross-sorption of Fluorescent Antisera to Each Strain.** MORRIS GOLDMAN AND NEVA N. GLEASON, Communicable Disease Center, Atlanta.

Aliquots of fluorescent antisera to two strains of *E. histolytica* and one of *E. hartmanni* were sorbed individually with about 1 million amebae of each strain. The sorbed antisera were then used to stain the three ameba strains in a standardized manner. Staining results were evaluated by microfluorimetric means. The results showed that the staining of *E. hartmanni* by homologous antiserum was not influenced by sorption with either strain of *E. hystolytica*, and conversely, that *E. hartmanni* amebae did not reduce the staining ability of the two antisera to *E. histolytica* for their homologous amebae. Among the *histolytica* strains, one antigen complex was common to both, but another one was found only in the more virulent of the two strains.

53. **Studies on *Myxosoma cerebralis* (Hofer) Plehn (Protozoa: Myxosporidea) the Cause of Whirling Disease of Trout.** G. L. HOFFMAN, C. E. DUNBAR, Department of the Interior, Bureau of Sport Fisheries, Kearneysville, West Virginia, and A. D. BRADFORD, Benner Spring Fish Research Station, Bellefonte, Pennsylvania.

This disease, apparently of central European origin, has more recently appeared in Russia, Italy, and the United States. Very young rainbow and eastern brook trout are most seriously affected. The trophozoites invade and erode cartilage causing malfunction of the organ of equilibrium which results in hectic, tail-chasing, whirling when disturbed. Other symptoms include crooked spinal columns, gaped mouths, sunken heads, and black tails. The multinucleate trophozoites grow and produce many pansporoblasts each of which produces several spores in about four months.

Whirling disease appeared at a Pennsylvania trout hatchery in 1956; one pond of fish was affected. The epizootic became widespread in 1957 and 1958 at which time a tentative diagnosis was made by Dr. S. F. Snieszko, USFWS. It was verified histologically by Dr. E. M. Wood, Seattle, Washington. In the spring of 1960 an attempt was made to clean and disinfect the hatchery using calcium cyanamide (1/10 lb. per sq. ft.) in the dirt ponds and chlorine gas (max. 300 ppm) in the spring water source. There has not been a recurrence of the disease in the 1961 fingerlings.

In 1960 Mr. Fred Howard discovered the disease at the National Fish Hatchery, Lamar, Pennsylvania, which is on the same watershed. There were relatively few fish affected and it is believed that only one series of ponds is contaminated.

During 1959–1961 we exposed early feeding rainbow and brook trout fry to the spores. Some of the fish developed whirling symptoms, but we could not verify the infections histologically.

54. **Competition Between Glucose and Galactose in the Culture Form of *Schizotrypanum cruzi*. Preliminary Report.** L. G. WARREN, Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela.

A comparative study of the incorporation of C 14 labeled glucose and galactose by *Schizotrypanum cruzi* during short periods of incubation (2 minutes) indicates that uptake of glucose is practically independent of concentration, whereas galactose uptake is proportional to the substrate concentration.

A study has been made of the interaction between these two substrates during the incorporation process. Glucose inhibited the uptake of galactose in glucose: galactose ratios as low as 1: 50. On the other hand, galactose did not inhibit the incorporation of glucose in ratios as high as 1: 1.

These results suggest that glucose and galactose share a common metabolic pathway or a common site of transport across the cell membrane.

55. **The Stability of a Strain of *Leishmania donovani*.** JOHN GRUN, Rutgers, The State University, New Brunswick, N. J.

The intercellular protozoan parasites used in this study were from the Khartoum strain of *Leishmania donovani* brought into this country by Meleney in 1943. Since then the integrity of this strain has been maintained by passages in the golden hamster, *Mesocricetus auratus*.

Attempts to select a strain of *L. donovani* more infectious for the mouse were made over a period of 3 years by passages involving the hamster and mouse.

For the passages in the mouse, old and young, males and females of Swiss Albino and CF_1 mice were surveyed. Various routes of inoculation and mass transfers of parasites were employed in order to: (1) vary the host response; and (2) select for possible mutants which might lead to a much greater protozoan population density in the mouse.

The spleen was generally the organ used from the donor animal as the parasite source; however, in some special cases the liver was the organ of choice.

In spite of careful selection of mouse donors and the use of hamsters to increase parasite numbers so that massive transfer could again be effected, it was evident that no marked increase occurred in the infectivity of this strain for the mouse.

Since the hamsters sub-inoculated from these later mouse passages showed typical infections and mortality times, the conclusion was reached that no change took place in the strain of parasites as a result of the several mouse passages.

(This investigation was supported by a research grant (E-92) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health).

56. (Withdrawn by authors.)

57. **A Comparative Study of Protein Fractions of Acanthocephala by the "Fingerprint" Technique.** T. T. DUNAGAN, Purdue University, Lafayette.

Since the time Ingram first introduced his "fingerprint" technique for the separation of normal and sickle cell globins, a variety of other applications of the technique have been introduced. The study of thermophilic bacteria flagella is one such example. The obvious differences that appeared in the latter prompted the author to apply this technique to the separation of species of *Neochinorhynchus*. After isolation, protein samples were digested at 37 C with trypsin at pH 8.0 in a pH-stat using 1 percent solutions of recrystallized enzyme. When digestion was completed, the pH was adjusted to 6.5 with 0.02 N HCl and the complete reaction mixture centrifuged. The resulting supernatant was lyophilyzed and prepared for electrophoresis which was continued for 2.5 hours at 1000 V. The peptides were further separated by ascending chromatography before development. The maps so obtained for *N. emydis* and *N. pseudemydis* are easily separated. Positively charged peptides formed more consistent patterns than those negatively charged. Some variation in certain peptides was obtained from one sample to another but throughout the samples tested, a consistent basic pattern occurred. Application of this technique to physiological races of certain species may help define their status more adequately. (This work was completed during a predoctoral fellowship, No. EF 9066, from the National Institutes of Health.)

58. *Mediorhynchus grandis* **(Acanthocephala: Gigantorhynchidae) in a Wild Turkey of South Dakota.** ERNEST J. HUGGINS AND CARL F. DAUMAN, South Dakota State College, Brookings.

A wild turkey, *Meleagris gallopavo merriami*, shot in the Black Hills of South Dakota (Fall City County) in the fall of 1958, harbored in its small intestine three female specimens of *Mediorhynchus grandis* Van Cleave, 1916. This is believed to be the first report of a thorny-headed worm from wild turkey anywhere, and the first report of a natural infection from turkeys (domestic or wild) in North America. Price (1929, J. Parasit. **15**: 290) reported larval forms of *Oncicola canis*, a parasite of dogs, in the esophageal lining of domestic turkey poults in Texas. Evidently, the only other record is that of Meyer (1932–3, Bronn's Klassen und Ordnungen des Tierrichs, pp. 183 and 355), in which he reported four specimens of *Mediorhynchus* (=*Empodius*) *giganteus* from a turkey in German East Africa.

Van Cleave (1947, Proc. Helm. Soc. Wash. **14**: 55–58) and Ward (1950, J. Tenn. Acad. Sci. **25**: 242–243) suggested that among the acanthocephalid worms having the strongest opportunity of becoming established in poultry are members of the genus *Mediorhynchus*. Various passerine birds have been reported as hosts of *M. robustus* and *M. papillosus*, and the latter was also found in a prairie chicken; *M. grandis* has been reported from the meadowlark, crow, and several species of grackles and blackbirds (Van Cleave, 1947, J. Parasit. **33**: 297–315). Experimental work by Moore (1941, J. Parasit. **27** (Supp.): 34) indicated that grasshoppers may serve as the intermediate host for *M. grandis*. Grasshoppers of Moore's experimental genera (*Chortophaga, Orphuella,* and *Arphia*) occur in the Black Hills. It is possible

that our turkey acquired its infection by ingesting an infected grasshopper on range frequented by one or more of the kinds of birds listed above.

59. **A Preliminary Study of the Histopathology of Acanthocephala in the Vertebrate Intestine.** W. L. BULLOCK, University of New Hampshire.

There are numerous references in the literature to the pathological effects of acanthocephalan parasites. Some of these indicate that even a few worms in a bird or fish may be serious or even fatal. However, heavy infections in some of these hosts often appear to do little obvious harm. In order to study in detail the relationship between some of these Acanthocephala and their hosts, a preliminary survey was made of the histopathological aspects of several different parasite and host species.

An acanthocephalan species with a small proboscis such as *Neoechinorhynchus cristatus* or *Atactorhynchus verecundus* appears to destroy only the epithelium and a small portion of the underlying lamina propria. These effects are limited to the site of attachment. There is little evidence of any extensive tissue damage or cellular reaction. Other worm species, such as *Polymorphus* sp and *Pomphorhynchus bulbocolli*, penetrate deeply through the submucosa and even the muscularis. These worms set off a marked connective tissue reaction with the development of a considerable fibrous capsule around the proboscis and neck. The most serious reaction appears to be with parasites such as *Acanthocephalus* sp. The fairly large proboscis penetrates deeply into the submucosa and there is a pronounced connective tissue reaction. This species appears to change its location in the host intestine. These factors combine in heavy infections to markedly interfere with the metabolism of the digestive system of the host.

60. **Studies on Ocular Trematodiasis. I. Marine Acquired Philophthalmiasis.** LAWRENCE R. PENNER AND BERNARD FRIED, University of Connecticut, Storrs.

Among the more than 10 kinds of cercariae found in studies on the trematode parasites of the most abundant marine melaniid gastropod along the Florida Gulf Coast, *Batillaria minima* (Gmelin) is one which readily forms flask-shaped cysts on the bottoms of isolation containers and in the host snails' feces. These when fed encysted or excysted to a variety of avian hosts, including the Mute Swan, Southern Chachalaca, Western Gull, pigeons and domestic chicks, develop to maturity under the nictitating membrane at variable rates.

Examinations of 20 mice and rats 9 to 14 days after exposure of 10 to 100 larvae via the esophagus, intraocularly, or through a slit made in the palate, were all negative.

Avian hosts were exposed successfully via the esophagus, median slit, and by placing larvae directly under the nictitating membrane although cloacal exposures were unsuccessful.

Adult flukes are related to the *lucipetus* group of 4 of the 23 described species in the genus *Philophthalmus* in having follicular rather than tubular vitellaria.

Avian hosts found naturally infected with *Philophthalmus* in the host snail's environment include the Yellow Crowned Night Heron, Laughing Gull, Willet, and Royal Tern.

The cercariae are closely allied to those of known species of *Parorchis* and *Cloacitrema* but the flask-shaped metacercarial cysts readily separate members of the genus *Philophthalmus* from the round to oval cysts of the others. Cercariae of this species of *Philophthalmus* apparently can infect directly but rapid encystment of pipetted cercariae invalidates quantitative data.

(This investigation was supported in part by Grant E-740 from the National Institutes of Health.)

61. **Studies on Ocular Trematodiasis. II. Growth of *Philophthalmus sp.* In Vivo and on the Chorioallantois of Chicks.** BERNARD FRIED AND LAWRENCE R. PENNER, University of Connecticut, Storrs.

Metacercariae of a *Philophthalmus* sp. obtained from naturally infected *Batillaria minima* (Gmelin) develop to maturity under the nictitating membrane of avian hosts. Swallowed larvae often fail to reach the orbit as compared to those entering via median slit or directly. Thirteen (46.4 percent) of 28 chicks exposed esophageally to 10 metacercariae each and examined 9 to 14 days later, were positive for 38 adults (13.6 percent). Forty-seven (97.9 percent) of 48 chicks given 10 larvae each by direct inoculation into the median slit were positive; 195 worms (40.6 percent) were recovered. Direct inoculation under the nictitating membrane succeeded in 11 of 12 chicks. Larvae inoculated into 1 eye sometimes migrated to the other eye.

The growth pattern was determined by intraocular or median slit inoculations of 1 to 10 larvae into 65 chicks. Flukes collected and measured daily from 2 to 65 days following exposures showed slow growth until the 5th day (average length from 0.76 mm to 1.00 at 5 days), became rapid (reaching 2.75 to 3.25 mm) until about the 20th day and when fertilization

occurred, continued growing at a slower rate (4.00 to 4.50 mm at 55 days). Unimetacercarial infections suggest that self-fertilization does not occur. Flukes not fertilized fail to grow beyond the 20-day level.

Oligoseptic metacercariae when placed on the chorioallantois of 7- to 10-day chick embryos differentiated and grew paralleling the ocular site. Several transfers 7 and 14 days later allowed survival with growth lagging 25 percent behind normal. Specimens became ovigerous past the 14th day but before the 20th day. (This investigation was supported in part by Grant E-740 from the National Institutes of Health.)

62. **The Protozoa as Parasites of Amphibia. I.** A. C. WALTON, Knox College, Galesburg, Illinois.

The following records of the presence of protozoan parasites in amphibian hosts have been added to the catalog of Parasites of Amphibia since the 1955 listing.—*ACRIS CREPITANS* (U.S.A.) :*Nyctotherus cordiformis, Opalina obtrigonoidei, Tritrichomonas augusta: AMBYSTOMA TIGRINUM* (U.S.A.): *Eimeria grobbeni, E. ranarum; ANEIDES FLAVOPUNCTATUS* (U.S.A.): *Cytamoeba bacterifera, Hexamita ovatus; ANEIDES LUGUBRIS* (U.S.A.): *Cytamoeba bacterifera; ANURANS* of Lauter, 1960 (Louisiana, U.S.A.): *Chilomastix caulleryi, Hexamita intestinalis, Karotomorpha swezyi, Monocercomonas batrachorum, Monocercomonoides elegans, M. nelolonthae, Octomastix batrachorum, Octomita neglecta, Retortomonas dobelli, Trepomonas agilis, Trimita parva, Tritrichomonas augusta, T. batrachorum, Trypanosoma karyozenkton, T. rotatorium, Urophagus intestinalis; BATRACHOSEPS ATTENUATUS* (U.S.A.): *Cytamoeba bacterifera; BOMBINATOR IGNEUS* (India): *Nyctotherus cordiformis; BUFO AMERICANUS* (Canada): Haemogregarines of Fantham et. al., 1942; *B. CINERITUS* (India): *Nyctotherus cordiformis; B. MARINUS* (Costa Rica): *Dactylosoma* sp? of Ruiz, 1959; *B. MARINUS* (Venezuela): *Haemogregarina legeri, Toxoplasma serpai; B. MELANOSTICTUS* (India): *Nyctotherus bufonis, N. macropharyngeus; B. MELANOSTICTUS* (Viet-Nam): *Balantidium* sp? of Boisson, 1957; *B. VIRIDIS* (Poland): *Tritrichomonas augusta; DICAMPTODON ENSATUS* (U.S.A.): *Cytamoeba bacterifera, Tritrichomonas augusta, Vahlkampfia* sp? of Lehmann, 1960; *ENSATINA ESCHSCHOLTZII* (U.S.A.): *Cytamoeba bacterifera, Vahlcampfia* sp? of Lehmann, 1960; and *HYLA REGILLA* (U.S.A.): *Opalina* sp? of Lehmann, 1960.

63. **The Protozoa as Parasites of Amphibia. II.** A. C. WALTON, Knox College, Galesburg, Illinois.

MICRIXALUS OPISTHORHODUS (India): *Balantidium duodeni, B. gracile, B. helenae, Nyctotherus cordiformis, N. macropharyngeus, N. magnus, N. m. malabarica, Opalina ranarum; M. SAXICOLA* (India): *Balantidium duodeni, B. gracile, B. helenae, Nyctotherus cordiformis, N. macropharyngeus, N. magnus malabarica, Opalina corocoidea, O. ranarum; NECTURUS MACULOSUS* (U.S.A.): *Proteromonas* sp? of Harris, 1954; *NOTOPHTHALMUS VIRIDESCENS* (U.S.A.): *Eimeria longaspora, E. megaresidua; PHILAUTUS* sp? (India): *Cepedea philauti, C. virgula, Nyctotherus cordiformis, Zelleriella froilanoi; PSEUDACRIS NIGRITA TRISERIATA* (U.S.A.): *Opalina obtrigonoidea; RANA A. AURORA* (U.S.A.): *Entamoeba ranarum, Haemogregarina aurorae, Tritrichomonas augusta; R. BREVICEPS* (India): *Nyctotherus breviceps, N. cordiformis, N. macropharyngeus, N. magnus, Protoopalina* sp? of Uttangi, 1958; *R. B. BOYLII* (U.S.A.): *Cepedea* sp? of Lehmann, 1960, *Haemogregarina boylii, Karolysus sonomae, Nyctotherus cordiformis, Opalina* sp? of Lehmann, 1960, *Trypanosoma boylii, Vahlkampfia* sp? of Lehmann, 1960; *R. C. CANCRIVORA* (Viet-Nam): *Balantidium* spp? (2) of Boisson, 1957, *Nyctotherus macropharyngeus, N. vesiculatus; R. CATESBIANA* (Canada): Haemogregarines of Fantham et al., 1942; *R. CLAMITANS* (U.S.A.): *Haemogregarina* sp? of Sanders, 1928; *R. CYANOPHYLCTIS* (India): *Balantidium duodeni, Nyctotherus cordiformis, N. magnus; R. CURTIPES* (India): *Balantidium helenae, Nyctotherus cochlearis, N. cordiformis, N. curtipes, N. kalii* (in tadpoles only), *N. macropharyngeus, N. magnus, Opalina ranarum; R. LEPTODACTYLA* (India): *Balantidium duodeni, B. helenae, Nyctotherus cordiformis, N. macropharyngeus;* and *R. LIMNOCHARIS* (India): *Balantidium duodeni, B. gracile, Nyctotherus limnocharis, N. magnus, Opalina corocoidea, O. ranarum.*

64. **The Protozoa as Parasites of Amphibia. III.** A. C. WALTON, Knox College, Galesburg, Illinois.

RANA PIPIENS (Canada): Haemogregarines of Fantham et al., 1942; *R. PIPIENS* (U.S.A.): *Chilomastix caulleryi, Entamoeba ranarum, Haemogregarina clamatae, H.* sp? of Failey, 1948, *H.* sp? of Fowler, 1946, *Nyctotherus cordiformis, Retortamonas dobelli, Trepo-*

monas agilis, Tritrichomonas batrachorum, Trypanosoma inopinatum, T. pipientis; R. SYLVATICA (U.S.A.): *Opalina obtrigonoidea, Trypanosoma pipientis, T. rotatorium; R. TEMPORARIA* (India): *Balantidium gracile, B. helenae, Nyctotherus macropharyngeus, N. magnus, N. m. malabarica, Opalina corocoidea, Tritrichomonas augusta; R. TIGRINA* (India): *Eutrichomastix batrachorum, Nyctotherus magnus; R. T. RUGULOSA* (Viet-Nam): *Balantidium helenae saigonensis, B. rotundum major, B. r. minor, Cepedea dracunula, Nyctotherus vesiculatus, Opalina tigrina, Protoopalina stevensoni saigonensis; R. WARSCHEWITSCHII* (Costa Rica): *Trypanosoma rotatorium; RHACOPHORUS LEUCOMYSTAX* (Viet-Nam): *Cepedea dimidiata saigonensis, Opalina cristulata; SALAMANDRA ATRA* (Europe): *Eimeria* sp? of Rudovsky, 1926; *SALAMANDRA MACULOSA* (Europe): *Eimeria salamandrae; TARICHA GRANULOSA* (U.S.A.): *Hexamita ovatus, Karotomorpha swezyi, Tritrichomonas augusta; T. G. TWITTYI* (U.S.A.): *Trypanosoma ambystomae, T. barbari, T. cryptobranchi, T. diemyctili, T. granulosae, T. tritonis; T. TOROSA* (U.S.A.): *Nyctotherus cordiformis; TRITURUS CRISTATUS* (Czechoslovakia): *Trichodina urnicola bohemica, T. u. typica; T. PALMATUS* (Europe): *Eimeria canaliculata, E. sphericum; T. TAENIATUS* (Czechoslovakia): *Trichodina urnicola taeniatus; T. VULGARIS* (Europe): *Eimeria sphericum;* and *UPERODON* (=*Cacopus*) *SYSTOMA* (India): *Balantidium helenae, Nyctotherus cacopusi, N. cordiformis, N. systoma, Opalina japonica.*

65. **Comments on** *Balantidium "suis"*. D. H. WENRICH, University of Pennsylvania, Philadelphia.

McDonald (1922, Univ. of Calif. Pub. Zool. **20**: 243–300) described *Balantidium "suis"* n. sp. from swine as having an elongated body, wider anterior to the equator, with longer and more laterally placed peristome and sausage-shaped macronucleus one-half body-length, in contrast to *B. coli* with oval body, more anteriorly placed peristome, and shorter and wider macronucleus.

By measuring 50 or more balantidia from each of 30 pigs (formalin fixation) I found average length: width ratios from 1.35 to 2.25. As reported by McDonald, Hsiung (1938, Zeitsch, Parasitenk. **10**: 108–131) and others, some natural populations appeared to be entirely *B. "suis"*-like, others entirely *B. coli*-like, the rest more or less intermediate. Hsiung considered that balantidia with L: W ratios of 1.90 or higher were *B. "suis"*; 26 of my groups had 2 to 94 percent of individuals with a ratio of 1.90 or higher. "Terminal" peristomes were uncommon; few animals had greatest width anterior to the equator. In a population from man (formalin fixation), 18.7 percent of 190 ciliates had L: W ratios of 1.90 or higher, but none had longer, slender macronuclei.

Hsiung, and Pick-Levontin and Cheissin (1940, Zool. Zhurnal. **19**: 99–118) established clones of *B. coli* and *B. "suis."* Both types varied greatly in body form, less in macronuclei. Both studies resulted in the conclusion that *B. "suis"* is a variety, not a species. Hsiung suggested that intermediate characters resulted from crosses between the two varieties. This attractive idea needs experimental verification. Like Pick-Levontin and Cheissin, I found "giant" forms of *B. coli*, which may be hyperploid individuals.

66. **Tumors in an Axenically Grown Species of Microannelid,** *Enchytraeus fragmentosus* **Bell, 1959.** D. JOANNE FERRAL, ELLSWORTH C. DOUGHERTY, AND CHRISTIAN F. HOLINKA, University of California, Berkeley.

We have observed tumorous *E. fragmentosus* under axenic conditions only. Knobby excrescences were first found (by D.J.F.) in February 1960 on an adult-sized worm axenized from axenic culture by antibiotic treatment and put into a crude (oligidic) liquid medium (dilute malted milk + 2 horse liver preparations + glucose), wherein neither obvious development nor fragmentation ensued. After 20 days numerous tumors studded the length of the worm, which died between 76 and 84 days after isolation. Recently, with sustained axenic cultivation realized in an oligidic semi-solid medium (on slants of 1½ percent Difco Nutrient Agar supplemented with heated liver extract—Dougherty and Solberg, *Nature,* in press), we find tumors relatively frequently—but, so far, only on worms trapped (at least temporarily) in free liquid (i.e., when enough is initially present, or subsequently accumulates, to form a meniscus at the slant's base). Tumors may appear on a fragment isolated from a tumor-free tube (shortest time: 5 days) or on groups of fragments, or of partly grown worms, descending from an ostensibly non-tumorous individual that had matured from an isolated fragment; even essentially adult-sized worms appear tumor-prone. In afflicted enchytrei, development typically stops (exception: 1 large tumorous worm fragmented into 2). Recent observations suggest tumors to be deliberately inducible if worms are submerged in added water (e.g., the 5-day period referred to). Current efforts are to seek: (1) a simpler medium; and (2) fur-

ther elucidation of tumor-inducing conditions; and to complete: (3) initial histological studies. (Supported, in part, by N.S.F. grants G-6018, -13138, and -15034.)

67. **Sporozoan Parasites of Sea Herring.** CARL J. SINDERMANN, U. S. Bureau of Commercial Fisheries Biological Laboratory, Boothbay Harbor, Maine.

As part of a continuing investigation of marine fish diseases, sporozoan parasites of sea herring, *Clupea harengus*, have been studied. Five species have been recognized: *Kudoa clupeidae* and *Ceratomyxa acadiensis* (Myxosporidia); *Eimeria clupearum* and *Eimeria sardinae* (Coccidia); and *Plistophora sp.* (Microsporidia). *Kudoa clupeidae* has been reported previously (Sindermann, 1957, J. Parasit. 43: 42–43) as an intramuscular parasite with a discontinuous distribution in immature herring of the western North Atlantic. *Cerotomyxa acadiensis* has been found in the gall bladders of several marine fish species from Canadian Atlantic waters, and is reported here as an occasional parasite of immature and mature sea herring. Two species of *Eimeria* occur in herring, although neither have been reported previously from the western Atlantic. *E. sardinae* occurs only in the testis, occasionally in great numbers in individual fish. This parasite has been reported to cause castration of European sardines (Santos-Pinto, 1957, Univ. Lisboa, Revista 5: 5–9). *E. clupearum* is a common parasite of the liver, especially of adult herring. *Plistophora sp.*, a microsporidian, was found in intramuscular cysts adjacent to the body cavity of very young herring, but has not been seen in fish over 9 months old.

Samples of herring of all sizes, distributed geographically from New Jersey to the Gulf of Saint Lawrence, have been examined yearly since 1955 for sporozoan and other parasites. Incidences of the Sporozoa mentioned in this paper have been less than 25 percent except for *Kudoa clupeidae*, which may occur in up to 80 percent of 1-year-old herring from certain coastal areas. Marked year to year variations have been noted only for *Kudoa* in which a continued downward trend in incidences has occurred, but it may well be that longer-term studies will disclose periodic characteristics of this and other parasites of marine fishes.

68. **Coccidial Parasites in the "Wrong Host" Animal.** J. C. LOTZE, R. G. LEEK, W. T. SHALKOP, AND R. BEHIN, (Present address: Johns Hopkins University, Baltimore, Md.) Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

For 4 years, cross-infection studies were conducted with 2 to 4 species of coccidia of caprine and ovine origin using young goats and sheep. These animals had been raised helminth-free and were either coccidia-free or had extremely mild coccidial infections of unknown origin. Failures seemingly resulted in attempts to infect 6-month-old goats with mixtures of sporulated oocysts of *Eimeria intricata, E. ninaekohlyakimovae, E. faurei,* and *E. arloingi* of ovine origin during one year's study and in attempts to infect 4-month-old sheep with mixtures of sporulated oocysts of *E. ninaekohlyakimovae* and *E. arloingi* of caprine origin in the next year's study. None of these experimental hosts showed outward evidence of infection by coccidia and none was slaughtered for examination of internal changes. Evidence from fecal examinations did not indicate that oocyst production was taking place in these animals as a result of the inoculations.

In studies during the next 2 years, sheep and goats were inoculated with mixtures of sporulated oocysts consisting of *Eimeria faurei, E. arloingi* and *E. ninaekohlyakimovae* of ovine origin. Certain sheep and goats, which had received comparable numbers of the oocysts, were slaughtered on schedule and internal changes noted. Two-month-old goats and sheep were used in one study and 7-month-old sheep and goats were used in the other. Deaths apparently from coccidiosis occurred among the sheep in both age groups and among the younger goats. Schizonts and sexual stages of coccidial parasites were found only in the sheep; schizonts only were found in the goats. The schizonts were more numerous in the sheep than in the goats and more numerous in the younger goats than in the older ones. The evidence indicated that the schizonts in the goats underwent degeneration after certain periods of growth and the merozoites formed in the larger schizonts did not appear to be mature. Severity of changes in the hosts, such as hydropic degeneration of visceral fat, edema of the mesenteries, and thickening of the intestines, were correlated with the number of schizonts found in the host animals.

69. **Bacteria-free Suspensions of *Eimeria acervulina* Sporozoites and the Effect of Antibiotics on Excystation.** DAVID J. DORAN AND MARION M. FARR, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

Bacteria-free suspensions of *Eimeria acervulina* sporozoites were obtained by treating

400,000 to 670,000 oocysts per ml of antibiotics (penicillin G, 3,100 units; streptomycin sulfate, 6,200 units; bacitracin, 770 units) for 4 days, then releasing the sporocysts by aseptic grinding with a mortar and pestle and finally treating them with sterile 0.25 percent trypsin 1:300 in 5 percent chicken bile at pH 7.3 to 7.6 (See abstract 102). The percentages of excystation were not significantly different from those obtained from sporocysts released from untreated oocysts. When sporocysts that had been mechanically released were treated with the same amounts of antibiotics, many of the sporozoites were damages and the percentages of excystation were less than one-half that of untreated sporocysts or sporocysts released from treated oocysts.

70. **Parasitologic Surveys in Cali, Departamento del Valle, Colombia. X. Enterobiasis in School Boys of Barrio Siloé, Cali, Colombia.** G. RAFAEL BONFANTE* AND ERNEST CARROLL FAUST,** Department of Preventive Medicine and Public Health, Faculty of Medicine, University of Valle, Cali, Colombia.

To determine the infection rate of *Enterobius vermicularis* in the boys of Barrio Siloé, a random sampling in the primary school of this ward of Cali was studied by Scotch tape anal swabs during 1959. The boys consisted of 31 who were 6 to 7 years of age; 81, 8 to 9 years; 53, 10 to 11 years; and 20, 12 to 13 years. This represented approximately 35 percent of all boys of these age groups in the ward. The swabs were taken at 8 A.M. as soon as the pupils had reached the school and were brought to the Parasitology Laboratory of the Department for examination. Swabs were made on successive school days. One hundred eighty-five boys provided seven swabs each, the data on which this analysis is based.

A total of 89 (48.0 percent) were diagnosed positive, 14 (45.1 percent) of the youngest group, 41 (50.6 percent) of the second group, 27 (50.9 percent) of the third group and 7 (35.0 percent) of the oldest group. Cumulatively, the total diagnosed incidence was discovered on first and 6 successive examinations as follows: 36 (19.3 percent, 15 (8.1 percent, 9 (4.8 percent), 13 (7.0 percent), 8 (4.3 percent), 5 (2.7 percent) and 3 (1.8 percent). Eight examinees each provided positives on all specimens, 3 each had 6 positives, 4 each had 5 positives, and the remaining 74 were positive a lesser number of times, often not in successive swabs. Complaint of pruritus ani was voiced by 36.0 percent of the diagnosed positives and 37.5 percent of the negatives, suggesting negative correlation between the symptom and the infection. However, 6 of the 8 positives with 7 positive swabs and with recorded large numbers of eggs on each tape specimen, were among those who complained of anal irritation.

71. **The Development of *Nematospiroides dubius* in an Abnormal Site in the White Mouse and Some Apparent Effects on the Serum Protein of the Host.** JOSEPH W. LEPAK AND VERNON E. THATCHER, University of Texas Medical Branch at Galveston.

White mice were given subcutaneous infections of third-stage larvae of *Nematospiroides dubius*, which normally develops in the intestinal tract. It was found that these nematodes did not migrate extensively within the body, but rather they became encysted in the subcutaneous tissues and underlying musculature near the site of infection. Development of the worms proceeded to the pre-adult stage in about 10 days. The worms gradually became encysted until at 30 days a well-defined vascularized cyst was present. The worms remained alive within these fibrous cysts for at least 43 days.

Electrophoretic studies were made on the serum protein of these and other mice. Preliminary results appear to indicate that mice with infections of mature worms resulting from larvae given orally do not differ markedly from uninfected controls. On the other hand, there appears to be an increase in certain serum fractions in the mice infected subcutaneously. The work was supported by National Institutes of Health Training Grant 2E-66 and National Science Foundation Grant G-4088.

72. **A New Series of Pyridines with Anthelmintic Activity.** ROBERT B. BURROWS, GEORGE R. HUNT, AND WILLIAM G. LILLIS, The Wellcome Research Laboratories, Tuckahoe, N. Y.

A number of quaternary pyridines were found to have activity against *Syphacia obvelata* in mice. Two of these compounds were particularly effective against *Syphacia* at low dosage levels and were tested against various helminth infections of dogs, cats and monkeys. One compound was not markedly active against these helminths, but the other demonstrated a wide spectrum of activity, giving good to excellent clearance against hookworms, roundworms,

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whipworms and several other nematodes.

The compound with the broadest anthelmintic spectrum, BW 48-236, was found to be active against *Syphacia obvelata* in mice; *Ancylostoma caninum* in dogs and cats; *Uncinaria stenocephala, Toxocara canis, Toxascaris leonina* and *Trichuris vulpis* in dogs; *Toxocara cati* in cats; and *Trichuris trichiura, Oesophagostomum apiostomum* and *Physaloptera* sp. in monkeys.

73. **A New Organophosphorus Systemic Insecticide for the Control of Larvae of *Oestrus ovis* L. in Sheep.** R. O. DRUMMOND, Ent. Res. Div., Agric. Res. Serv., U.S.D.A. Livestock Insects Investigations, Kerrville, Texas.

In preliminary trials, single oral drenches of Bayer 37342 (*O,O*-dimethyl *O*-(3,5-dimethyl-4-methylthiophenyl) phosphorothioate) at dosages of 50 and 100 mg per kg were 100 percent effective in ridding sheep of all three larval instars of *Oestrus ovis* L. At 25 mg per kg the compound was highly, but not completely, effective. Single treatments of 50 and 100 mg per kg in the feed were also 100 percent effective, but intramuscular injections of 25 and 50 mg per kg were less effective.

In tests with larger numbers of sheep, a single drench of 50 mg per kg was 100 percent effective against all instars, and 25 mg per kg was 100 percent effective against first instars, but not second and third instars.

Most bots were expelled prior to sacrifice and examination of the sheep, but a few were found dead in situ or in blind sinuses.

None of the sheep exhibited symptoms of phosphorus insecticide poisoning.

74. **The Anthelmintic Effect of a Sulfamoylphenyl Phosphorothioate on the Gastrointestinal Nematodes of Ruminants.** I. B. WOOD, J. E. EMRO, AND E. WALETZKY, Agricultural Division, American Cyanamid Company, Princeton, New Jersey.

Broad spectrum anthelmintic activity was obtained with phosphorothiotic acid, O,O-dimethyl-O-*p*-(dimethylsulfamoyl-phenyl ester (designated as American Cyanamid 38,023), against many of the economically important parasites of sheep. It was first shown to be an effective systemic insecticide in the mouse-*Aedes* test system (Hewitt, unpublished data) and later against cattle grubs.

Control and critical tests in sheep with experimental and natural infections were used to evaluate the efficacy of this compound against adult worms. Single oral doses of 60 mg per kg produced 98 percent or more elimination of the adult worms of *Haemonchus contortus* and of *Cooperia spp.;* 80 mg per kg gave the same control of *Ostertagia spp., Trichostrongylus axei,* and *T. colubriformis. Nematodirus spp.* were much less susceptible.

In sheep, this compound was relatively safe orally at 400 mg per kg with temporary partial anorexia of 2 or 3 days duration; 800 mg per kg was lethal. No signs of toxicity were observed grossly with oral doses of 200 mg per kg or less.

75. **Thiabendazole, A New Broad Spectrum Anthelmintic.** A. C. CUCKLER, Merck Institute for Therapeutic Research, Rahway, N. J.

Since there is a need for an anthelmintic with a wide range of antiparasitic action, a high degree of efficacy, a good margin of safety and with versatility of administration, research was conducted to discover and develop such a compound. Thiabendazole has promise of fulfilling to an extraordinary degree these requirements for a veterinary anthelmintic.

Thiabendazole [2,(4′-thiazolyl)-benzimidazole] is a stable, white crystalline compound with the empirical formula of $C_{10}H_7N_3S$ and a molecular weight of 201.3 (Brown et al, J. Am. Chem. Soc. **83**: 1764-1765, **1961**).

Studies in sheep, goats, cattle, swine, dogs, and chickens have revealed thiabendazole has a broad anthelmintic spectrum affecting numerous roundworms and certain tapeworms. This anthelmintic is particularly effective for roundworms belonging to the orders, Strongyloidea, Ascaroidea and Trichinelloidea. Efficacy studies in sheep, for example, indicate 14 species of the following genera of parasites are responsive to thiabendazole: *Trichostrongylus, Haemonchus, Ostertagia, Cooperia, Nematodirus, Bunostomum, Strongyloides, Chabertia, Oesophagostomum, Capillaria, and Trichuris.* The same or closely related parasites in cattle and goats also respond to this anthelmintic. In critical tests 8 non-treated control sheep harbored an average of 5700 worms, representing 11 genera. Thiabendazole in single oral dosages of 12.5, 25 or 50 mg per kg of body weight (4 sheep per group) removed 80, 86, and 98 percent of the worms, respectively. Thiabendazole was especially effective in removing immature parasites. Phenothiazine in single oral dosage of 500 mg per kg removed 11 percent

of the worms.

The safety, efficacy and utility of thiabendazole as an anthelmintic for veterinary use will be summarized.

76. **Effect of Thiabendazole upon Infections of *Trichinella spiralis* in Mice, and upon Certain Other Helminthiases.** W. C. CAMPBELL, Merck Institute for Therapeutic Research, Rahway, N. J.

Thiabendazole has been found effective against experimental trichinosis in mice and rats. Diet containing 0.1 percent thiabendazole was fed ad libitum to mice for 4 days prior to exposure to heavy infections of *Trichinella spiralis*, and for 2 days after exposure. All of the untreated control mice died within 6 days. There was no mortality in the treated group of mice, and no worms could be recovered from the intestine at autopsy, 7 days after exposure. In the same experiment there were no deaths in the group of mice receiving a diet of 0.05 percent thiabendazole, but small numbers of worms were present in the intestines at autopsy. Other experiments, using sublethal infections, have confirmed the prophylactic efficacy of thiabendazole against *T. spiralis*. A single oral dose of 250 mg per kg of body weight, administered to mice 1 day after exposure to *T. spiralis*, also prevented the establishment of an infection.

In addition, thiabendazole has been found efficacious, in relatively large doses, against 2 species of tapeworms. Rats experimentally infected with *Hymenolepis diminuta* were completely free of tapeworms after a single oral dose of thiabendazole at 300 mg per kg, or after feeding 0.3 percent of the drug in the diet for 7 days. However, administration of this concentration of the compound in the diet for 3 days before, and 4 days after, exposure to *H. diminuta*, did not prevent the establishment of infections. Experimental infections of *Raillietina cesticillus* in chickens were reduced aproximately 90 percent by administration of a diet containing 0.3 percent thiabendazole for 9 days, or by a single oral dose of 500 mg per kg. No prophylactic efficacy could be demonstrated when a diet containing 0.5 percent thiabendazole was fed to chickens for 2 days before exposure to *R. cesticillus*, and for 5 days after exposure.

Two helminthic infections failed to respond therapeutically to thiabendazole. Dogs with natural infections of heartworm (*Dirofilaria immitis*) were treated by intravenous infusion of thiabendazole hydrochloride solutions. Neither the circulating microfilariae nor the adult worms were killed by doses up to 150 mg per kg. Administration of a diet containing 0.4 percent thiabendazole, for 2 weeks, to mice infected with *Schistosoma mansoni*, did not elicit a detectable therapeutic response.

77. **The Effect of Thiabendazole upon *Ascaris* and *Stephanurus* Infections.** J. R. EGERTON, Merck Institute for Therapeutic Research, Rahway, N. J.

Since thiabendazole was found to have marked therapeutic effect upon immature forms of various gastrointestinal nematodes of ruminants, studies were undertaken to determine whether this anthelmintic could be used to prevent ascariasis and stephanuriasis.

Mice given single oral doses of 100,000 larvated eggs of *Ascaris suum* suffered average mortality of 62 percent (range 50 to 80 percent) within 10 days after infection. Surviving mice were autopsied and found to have severe haemorrhages in the lungs. Mice fed 0.1 or 0.3 percent concentrations of thiabendazole in their diet for 1 day preceding and 9 days after infection showed slight to marked decreases in mortality and lung pathology.

Swine fed rations containing 0.1 or 0.4 percent thiabendazole for 4 days before and 10 days after infection with 180,000 larvated *Ascaris* eggs showed reduced liver and lung pathology compared to untreated infected controls at autopsy on the 10th day after infection. Swine with natural or experimental *Ascaris* infections were fed rations containing 0.003 to 0.1 percent thiabendazole for perids of 3 to 14 weeks. Expulsion of adult worms from the treated animals was preceded by a decrease in worm egg production. Autopsy examinations for lesions and parasites showed this anthelmintic was highly effective in removing ascarids when fed at concentrations of 0.01 percent or higher.

Studies revealed thiabendazole prevents the embryonic development of *Ascaris* eggs in vitro. The concentration of drug required for complete inhibition of embryonic development is less than 1 ppm.

Limited observations indicated that swine fed thiabendazole and experimentally infected with *Stephanurus dentatus* larvae are protected from the liver pathology associated with stephanuriasis.

78. **Anthelmintic Activity and Metabolism of Ruelene Administered to Sheep.** J. A. TIMMERMAN, JR., H. F. TURNER, AND B. W. ARTHUR, Department of Zoology-Entomology, Auburn University, Auburn, Alabama.

P-32 labeled Ruelene (4-tert-butyl-2-chlorophenyl methyl methylphosphoramidate) administered orally to sheep was rapidly absorbed through the alimentary tract, metabolized, and eliminated chiefly in the urine as water-soluble degradation products. By 24 hours after treatment of sheep at 50 mg per kgm, 52 percent of the administered Ruelene equivalents was eliminated in the urine and 4 percent in the feces. Eight metabolites were isolated from the urine and four of these were characterized by column chromatographic and radiotracer techniques. Five and possibly six metabolites were isolated from several genera of helminths taken from the digestive tract of sheep sacrificed at 6 and 24 hours after treatment.

Ruelene formulated as a polymer reduced intestinal absorption, decreased the quantity of residues in the internal tissues, protected the phosphate from enzymatic degradation, and forced a larger percentage of the administered material to be eliminated in the feces. The polymer constituents also prevented the Ruelene from leaching from the feces when placed under natural weathering conditions for 20 days.

Twenty sheep were treated orally at 121.5 mg per kgm, with three formulations of Ruelene (wettable powder, liquid drench, Ruelene + Polymer, and Ruelene + Polymer mixed with feed), and 5 sheep served as untreated controls. Five days after treatment, the alimentary tracts were examined for internal parasites. These formulations were quite effective against *Haemonchus sp.*, *Trichostrongylus sp.*, *Osteragia sp.*, and *Trichuria sp.* Ruelene was not effective against *Nematodirus sp.*, and *Moniezia sp.* Also Ruelene was not effective against sheep nose bot (*Oestrum ovis* L.). These data on the anthelmintic activity of Ruelene formulations were substantiated under ranch conditions using E.P.C. counts as a criteria for measuring effectiveness. Weight gains of the treated sheep as compared to the untreated controls were significant at the 0.1 percent level.

79. **On the Ineffectiveness of Thiabendazole Against the Migrating Larval Stages of the Swine Kidney Worm** (*Stephanurus dentatus*) **in Rabbits.** J. E. ALICATA, University of Hawaii, Honolulu.

Thiabendazole (2-(4'-Thiazolyl)-benzimidazole) has been reported to possess significant anthelmintic activity for a variety of gastrointestinal parasites of livestock and to interfere with the migrating larval stages of ascarid and kidney worms in swine (Brown et al, 1961, J. Am. Chem. Soc., in press). Preliminary tests have been conducted by the writer to determine the prophylactic value of this drug in preventing the migration of kidney worm larvae and in reducing the pathology in the liver of experimentally infected rabbits. In this test, 3 groups (3 each) of rabbits were each administered orally 2000 infective kidney worm larvae. Groups I and II received the drug orally at the daily rate of 50 mg per kg and 150 mg per kg of body weight, respectively, and group III remained as untreated control. The required amount of the drug per day was dissolved in 2 cc of water and administered by stomach tube in 2 daily doses (early morning and late afternoon) of 1 cc each. The period of prophylactic treatment covered 4 days preinfection and 10 days postinfection. All the rabbits were sacrificed about 1 month postinfection. At necropsy the livers showed numerous superficial whitish tracts and fibrous parenchyma, and there was no noticeable difference in pathology between the treated and untreated animals. Artificial digestion of the livers of the 3 rabbits in each group revealed the following number of developing kidney worm larvae: Group I, 17, 19, 31; group II, 15, 20, 28; group III, 13, 16, 27. The larvae were approximately 4 to 6 mm long.

80. **Preliminary Observations on the Resistance of Horse Strongyles to Phenothiazine.** J. H. DRUDGE AND GEORGE ELAM, University of Kentucky, Lexington.

The failure of therapeutic doses of phenothiazine to alter post-treatment strongyle egg counts was a consistent observation in 9 tests on 3 local thoroughbred farms during 1960–61. In recent years all 3 farms have relied on periodic therapeutic doses of phenothiazine for strongyle control; none has used the low-level system.

Four commercial phenothiazine products, including 2 N. F. Green, 1 microfine, and 1 microfine and purified preparation have shown no apparent difference of activity in the present observations. Dosing has been at the rate of 2.5 gm per cwt; stomach tube administration has been the most common method.

The average pre-treatment strongyle EPG for the nine tests (50 mares) was 675 (range = 140–1,280) and the average EPG 2 weeks post-treatment was 665 (range, 190 to 1,260). Differential larval counts indicate that the great bulk of the resistant forms are small

strongyles. The numbers of large strongyle larvae in the pre-treatment cultures have been too small to permit a proper evaluation of the response of these forms; however, appreciable numbers of both *Strongylus vulgaris* and *S. edentatus* larvae were present in some of the post-treatment cultures.

Simultaneous evaluation of therapeutic doses of piperazine alone and combinations of piperazine and phenothiazine in comparable groups of animals on all 3 farms resulted in consistently high level reductions of post-treatment strongyle EPG and averaged 95 percent and 97 percent, respectively. (Approved for publication by the Director, Kentucky Agricultural Experiment Station, and supported in part by the Grayson Foundation, Inc.)

81. **The Action of Some Phenothiazine Derivatives Against the Free-living Stages of** *Nematodirus spathiger*. WILLIAM C. MARQUARDT, Veterinary Research Laboratory, Bozeman, Montana.

Nine phenothiazine derivatives were tested for their effects on the free-living stages of *Nematodirus spathiger*, strain KT. The compounds were all substituted on the 10-position; in two the sulfur was also oxidized, and in five there were substitutions on the 2-postion. Activity against free-living stages of *N. spathiger* was measured by the suppression in development, delay in hatching the reduced motility of the infective larvae. All compounds showed activity at concentrations of 10^{-3} M, and seven of the nine at 10^{-4} M. Minor differences were seen in the stages of development, such as early development or hatching, on which one compound or other exerted its effect. However, when compounds differing from one another in a single substituent were compared, no diffrences in activity were evident.

The following alterations in the structure were found to have no effect on activity: (1) oxidation of the sulfur, (2) addition of $-CH_3$, $-OCH_3$, $-SH_3$, or $-CF_3$ on the 2-position, (3) the configuration of 5- and 6-carbon aliphatic tertiary amines attached on the nitrogen and (4) inclusion of a piperazine moiety in the chain attached to the nitrogen.

It is concluded that the phenothiazine portion of the molecule is primarily responsible for the activity of the compounds. (This investigation was reported by the Western Regional Project, W-35, "Nematode Parasites of Ruminants.")

82. **Observations on the Efficacy of Three Phenothiazine Preparations on a Phenothiazine-Resistant Strain of** *Haemonchus contortus*. J. H. DRUDGE, Z. N. WYANT, AND GEORGE ELAM, University of Kentucky, Lexington.

In three experiments, utilizing a total of 21 lambs raised helminth-free, the efficacies of single doses of 3 phenothiazine preparations at 0.1 g per lb were compared against strain B *Haemonchus contortus* of Drudge et al (Am. J. Vet. Res. **18**: 317–325). The same lot of a commercial phenothiazine (N.F. Green) with an average particle size of 10.8 microns that was found in earlier tests (Am. J. Vet. Res. **20**: 670–6) to remove 55.4 percent of the males and 69.2 percent of the females of this strain of *H. contortus* was also used in the present studies. A second commercial N.F. Green preparation with an average particle size of 8.2 microns and a third product, a purified preparation with an average particle size of 17.0 microns were used. Particle size determinations were made in a Fisher sub-sieve sizer.

Lambs were experimentally infected with 2,500 larvae and treated approximately 40 days later. Drug administration was via dose syringe equipped with a Whitlock nozzle. Data on 24-hour fecal egg counts and worms recovered at necropsy on three untreated animals were used to estimate the number of worms in the treated animals before treatment. These estimates and the number of worms recovered at necropsy 1 week after treatment determined the removal efficacies.

Six lambs were treated with each preparation, and in the aggregate the average removal efficacies (percent) for the three products (in the foregoing order) against male and female *H. contortus* were 59.5 and 78.8, 57.8 and 80.3, and 79.2 and 92.8, respectively. The differences were not statistically significant; further testing is required to establish possible relationships among purity, particle size, and efficacy against this strain of *H. contortus*. Approved for publication by the Director, Kentucky Agricultural Experiment Station, and contributing to Southern Regional Project S-21, Gastro-Intestinal Parasites of Ruminants.)

83. **Comparison of Thiabendazole, Ruelene and Phenothiazine for Anthelmintic Activity in Sheep.** J. H. DRUDGE AND GEORGE ELAM, University of Kentucky, Lexington.

In two field tests, the efficacies of single oral doses of 2-(4′-thiazolyl)-benzimidazole (Thiabendazole, Merck Sharp & Dohme) at 100 mg per kg; 4-tert-butyl-2-chorophenyl methyl methylphosphoramidate (Ruelene, Dow) at 125 mg per kg; and phenothiazine (microfine, purified) at 550 mg per kg were evaluated by post-treatment reductions of worm egg counts.

For the first test, 20 ewe lambs having an average EPG of 1,880 were divided into three treatment and one control groups. Differential egg counts before treatment indicated that *Haemonchus* was dominant (86 percent) while the remaining eggs were distributed in decreasing order among *Ostertagia, Trichostrongylus, Cooperia,* and *Oesophagostomum. Nematodirus* and *Strongyloides* eggs were present in very small numbers. At 7 days post-treatment average changes (percent) of EPG (exclusive of *Strongyloides*) for the Thiabendazole, Ruelene, phenothiazine, and control groups were −99.99, −97.6, −80.3, and +19.8, respectively. These levels were maintained in weekly counts thereafter until the fourth week when moderate increases were recorded in all three treatment groups. *Moniezia* was not affected by any of the compounds as indicated by the continued presence of eggs in the post-treatment fecal samples.

In a second test of similar design, 30 breeding ewes having an average EPG of 250 were divided into 4 groups. *Haemonchus* was dominant (62.7 percent) in the pre-treatment EPG's, while eggs of *Trichostrongylus, Oesophagostomum, Ostertagia,* and *Cooperia* were present in decreasing order. *Strongyloides* made up 16.9 percent of the average EPG before treatment. At 7 days post-treatment, the average EPG (exclusive of *Strongyloides*) reductions (percent) for the Thiabendazole, Ruelene, phenothiazine, and control groups were −99.8, −94.2, −91.9, and −17.9, respectively. Changes in *Strongyloides* EPG were −97.5 percent, +18 percent, −21.3 percent, and +28.6 percent, respectively. (Approved for publication by the Director, Kentucky Agricultural Experiment Station, and contributing to Southern Regional Project S-21, Gastro-intestinal Parasites of Ruminants.)

84. **Synergistic Action between Phenothiazine and Piperazine-Carbon Disulfide Complex against Horse Strongyles.** J. H. Drudge, Z. N. Wyant, George Elam, and G. Rothenberger, University of Kentucky, Lexington.

The anthelmintic activity of single therapeutic doses of phenothiazine (2.5 gm per cwt) and piperazine (4.0 g base per cwt) when used separately may be generally characterized as follows: (1) both compounds are highly effective on small strongyles, (2) phenothiazine removes about 50 percent of the large strongyles, *Strongylus vulgaris* and *S. edentatus,* and (3) piperazine removes about 50 percent of *S. vulgaris* but is devoid of activity against *S. edentatus.*

Three critical tests were completed in which various dosage combinations of phenothiazine and the piperazine-carbon disulfide complex (Parvex, The Upjohn Company) were used. The drugs were administered by stomach tube and the animals were necropsied after a 7-day post-treatment fecal collection period.

The removal efficacies (percent) against small strongyles, *S. vulgaris* and *S. edentatus* were 100, 99.5, and 96.7, respectively, in one test on full therapeutic doses of phenothiazine (2.5 g per cwt) plus the complex (4.0 g base per cwt); 96.0, 98.1, and 96.8, respectively, in a test with phenothiazine at 1.25 g per cwt plus the complex at 4.0 g base per cwt; and 98.5, 100, and 52, respectively, in a test with phenothiazine at 1.25 g/cwt plus the complex at 2.0 g base per cwt. (Approved for publication by the Director, Kentucky Agricultural Experiment Station and supported in part by the Grayson Foundation, Inc.)

85. **Observations on the Use of Thiabendazole in Sheep and Cattle.** W. S. Bailey, D. L. Diamond, and D. F. Walker, Auburn University, Alabama.

A flock of 51 sheep was divided into four groups. One group received no anthelmintic. The other groups were given phenothiazine (24 gm per cwt), Dylox (73 mg per kg), or Thiabendazole (55 mg per kg). The drop in egg production following treatment was most pronounced in the sheep receiving Thiabendazole; next in those treated with Dylox; and about the same in those receiving phenothiazine and untreated. Worm counts were made (18 to 30 days following treatment) of three sheep from Thiabendazole group and two from each of the other groups. Except for one sheep with large number of immature worms, those treated with Thiabendazole had significantly fewer nematodes in the abomasum and small intestine than did the sheep of other groups.

Five of nine cows showing clinical evidence of parasitic gastritis were treated with Thiabendazole (55 mg per kg). Included in this group were all animals showing the most pronounced clinical signs of infection except for one that died before treatment was given. There was a marked drop in egg counts of all treated animals following treatment. Two of the cows were given a second treatment (88 mg per kg) 28 days after the first. Worm counts were made at necropsy of the five treated and three untreated animals. On the basis of comparisons of number of worms recovered, severity of abomasal lesions, weight gains in animals kept as long as 40 days and clinical response, Thiabendazole appears to offer considerable

promise in treating clinical parasitic gastritis of cattle.

86. **A Preliminary Report on the Effects of *Bacillus thuringiensis* var. *thuringiensis* Berliner on the Development of the Free-Living Stages of Some Cattle Nematodes.** H. CIORDIA AND W. E. BIZZELL, Regional Animal Disease Research Laboratory Substation, Agricultural Research Service, U. S. Department of Agriculture, Experiment, Ga.

Viable spores of *Bacillus thuringiensis* are presently used as biologic agent in the control of some insect pests of vegetable crops. Suppressive effects have also been reported on the development of houseflies in cattle feces after feeding bacillus spore-crystal powder as a feed additive.

Freshly voided feces from dairy calves experimentally infected with *Cooperia punctata, C. oncophora,* and *Ostertagia ostertagi* were used for egg cultures. These were made by placing 80 g of thoroughly mixed feces in individual paper cups and incorporating different concentrations of the spore material. Materials tested were Bakthane L-69 (Rohm and Haas Company, Philadelphia, Pa., concentration 7.5×10^{10} spores per g) and Thuricide (Stauffer Chemical Company, New York, N. Y., concentration 6.5×10^{10} spores per g). Third-stage larvae recovered from each culture were counted and identified after an incubation period of 10 days at 27 C.

The number of larvae recovered diminished as the concentration of spores was increased. The percentages of larval development, as compared to the numbers of larvae obtained from the control cultures, were as follows. 33.95, 26.61, 20.14, 6.61, 4.57, and 0.18 from cultures containing 4.69×10^7, 1.41×10^8, 2.34×10^8, 4.69×10^8, 9.38×10^8, and 1.88×10^9 spores per g of feces, respectively. The larvae of the three species appeared to be equally susceptible to the bacillus spore material.

87. **Observations on Parasitism of the Raccoon of Cape Island, S. C.** REINARD HARKEMA AND GROVER C. MILLER, N. C. State College.

Cape Island is one of the islands contained within the boundaries of the Cape Romain Wildlife Refuge and is located approximately 35 miles northeast of Charleston, S. C. In June 1960, opportunity was afforded by Mr. E. S. Jaycocks, Refuge Manager, to collect and examine 11 raccoons for parasites. A total of 13 genera of helminths was recovered with every host infected with one or more. The majority of the helminths were trematodes representing 5 families. The following is a list of these worms; in parentheses are represented the number of hosts infected and the approximate average number of specimens per infected host: NEMATODA: *Gnathostoma spinigerum* (1, 1), *Physaloptera rara* (9, 14), *Arthrocephalus lotoris* (1, 1); TREMATODA: Microphallidae: *Carneophallus* (*turgidus?*) (11, 766), *Gynaecotyla adunca* (8, 226), *Maritrema* sp. (1, 2); Heterophyidae: *Phagicola* spp. (8, 416), *Ascocotyle leighi* (3, 58); Dicrocoeliidae: *Lyperosomum sinuosum* (6, 138); Nanophyetidae: *Sellacotyle mustelae* (1, 6); Diplostomidae: *Fibricola* (*texensis?*) (5, 104), *Pharyngostomoides procyonis* (1, 9). It is interesting to note that two-thirds of the trematode species have been described as parasites of birds. They are members of families which have demonstrated non host-specificity. Another interesting observation is the absence, except for one specimen of *Arthrocethalus lotoris,* of parasites with a direct life-cycle.

88. **Developmental Stages in the Life Cycle of *Plagiorchis goodmani* (Trematoda: Plagiorchiidae).** H. H. NAJARIAN, The University of Texas Medical Branch, Galveston, and University of Michigan, Ann Arbor.

The experimental life cycle of *Cercaria goodmani* was established in the laboratory. Chicks and canaries served as definitive hosts. The adult worms live in the small intestine. Fluke eggs required 4 to 5 days incubation for development of miracidia, which had to be eaten by snails (*Lymnaea palustris*) for transformation into sporocysts and cercariae. Metacercariae developed both in tricopterous larvae and adults (*Limnephilus*), and occasionally in sporocysts within *L. palustris.* Adult worms developed in birds 11 days after feeding metacercariae. The natural vertebrate host is unknown.

89. **Studies on Virgulate Cercariae from *Nitocris dilatatus* Conrad and Their Entry into Arthropod Second Intermediate Hosts.** J. E. HALL AND A. E. GROVES, West Virginia University School of Medicine.

During 1959 and 1960, 1859 specimens of *Nitocris dilatatus* from the Cheat River, West Virginia yielded 9 species of virgulate xiphidiocercariae. Two of these have been identified as *Cercaria tremaglandis* and *C. adoxovirgula* Hall, 1960; 7 differ from species described in the

literature. Potential arthropod hosts were determined for 5 of the 9 species by exposing aquatic insects belonging to 8 different orders; limited attempts to follow the full course of the infection in the insects were unsuccessful.

Cercaria tremaglandis and an undescribed cercaria consistently entered ephemerid mayfly naiads. Fourteen naiads 8 to 12 mm long from an uninfested stream were exposed individually to 150 to 300 cercariae each for periods ranging from 15 minutes to 15 hours; they were fixed immediately following exposure and were subsequently sectioned and stained in thionin or a trichrome stain. Both cercariae enter the naiads through the distal portions of the gills or through the thin cuticle of the body-segment, leg, or mouthpart articulations. A host cellular response to entering and migrating cercariae was observed. Some mucoid material is discharged from the virgula organ as the cercariae attach to the body surface; the remainder is discharged soon after entry, usually prior to migration into the muscles and fat body. This mucoid may be surrounded by hos plasma cells as early as 1 hour after exposure. (This work is supported by U. S. Public Health Service Research Grant E-3356.)

90. **A Cercaria of the Haloporidae (Trematoda: Digenea) and the Affinities of That Family.** R. M. CABLE, Purdue University and Caraibisch Marien-Biologisch Instituut, Curaçao, N.A.

A biocellate, gymnocephalous cercaria has been found to develop in simple rediae in the marine snail, *Rissoina browniana*, in Curaçao. The body is spinose anteriorly. It contains yellow pigment and dense cystogenous glands but no penetration glands. The digestive system is well developed and the esophagus bifurcates at the posterior edge of the ventral sucker. The excretory system is stenostomate with primary pores on the sides of the tail. The bladder is saccate and evidently non-epithelial. The tail is simple and lost soon after emergence when the larva encysts to become a floating metacercaria with a thick, gelatinous outer cyst adapted to ingestion with vegetation. Collectively, features of the cercaria exclude it from any larval type for which a life history has been reported and persistent ones are in agreement with adult morphology in but one family of marine trematodes in the Caribbean region, viz, the Haloporidae, species of which parasitize herbivorous and omnivorous fishes. Furthermore, an immature worm with features of both the cercaria and adult haloporids, including their characteristic lymph channels, was recovered from the intestine of a surgeon fish (*Acanthurus hepatus*) 5 days after exposure to infection. The structure and development of the cercaria and features of its life history indicate that the haloporids, although resembling; the Lepocreadiidae and Acanthocolpidae in adult structure, are more closely related to the Haplosplanchnidae even though the one known cercaria of that family differs from the haloporid larva in having a rhabdocoele intestine, small excretory bladder, and an ornate tail. (Supported by National Science Foundation Grant No. G14691.)

91. **Some Trematodes from El Salvador.** ELMER C. HERBER AND ALFONSO TREJOS, Dickinson College, Carlisle, Pa., and University of El Salvador, San Salvador, C.A.

Six species of digenetic trematodes, mostly mature, were collected from 24 coots, *Fulica americana americana*, an undetermined shark, and a crocodile, *Crocodylus acutus acutus*, from El Salvador. Two species of larval trematodes from two different snails are regarded as new. Most of the species described are new locality records.

It was found that *Hyptiasmus arcuatus* has 33 pairs of penetration glands in an immature specimen. These glands could not be found in adults examined in another collection. The function of these glands is postulated.

In *Petalodistomum pacificum* no variation in the number of testis follicles of 10 on one side and 12 on the other side was observed. The original description noted some variation.

Acanthostomum americanus showed a definitely larger posterior testis than the anterior testis. An anal pore at the posterior extremity of each intestinal ceca is also noted. In the original description these observations were not pointed out.

Cercaria appendicata is described for the first time. It is a cystophorous cercaria with an anterior appendage of unknown function. Some flat ribbon-like extensions from the cyst-like center are also an enigma.

Cercaria porrilli is a new stylet cercaria from *Physa lacustris*. It has eight pairs of penetration glands. Feeding experiments from encysted stylet forms taken from dragon fly nymphs from the same area were unsuccessful.

92. **The Biology of *Nematobothrium texomensis* McIntosh and Self, 1955 (Didymozoidae), in the Buffalo Fishes of Lake Texoma.** J. TEAGUE SELF, LEWIS E. PETERS AND CLARENCE E. DAVIS, Department of Zoology, The University of Oklahoma, Norman.

Nematobothrium texomensis occurs in *Ictiobus bubalus, I. cyprinellus,* and *I. niger* in Lake Texoma and the Red River which it impounds. This worm is known also from buffalo fishes of other Mississippi basin streams as well as at least one Gulf Coastal stream.

A 12-months' study reveals that *N. Texomensis* appears first in buffalo fishes of Lake Texoma during November. At this time some are mature and producing eggs while others are immature being about 10 inches long. They have been recovered alive only from mature gonads, and with one exception, gravid ovaries. They have not been found in spawned ovaries except in a deteriorated condition. Some female hosts do not spawn, and resorb their eggs. So far as we can determine worms in these ovaries always die and deteriorate. Their remains consisting of dead eggs are found in many redeveloped ovaries.

Live worms are present between November and early August after which only dead ones are found until November. This plus inability to find larval forms in possible invertebrate hosts, plus their appearance in a fairly advanced stage of development in November, suggests that the fish migrate out of the lake into the tributaries where they are infected.

We have been unable to hatch eggs in water or by passing them through snails. Force hatching reveals an ameboid miracidium, which is not ciliated and has the crown of spines characteristic of the miracidia of the Hemiuridae as well as the Didymozoidae.

93. **Observations on *Aelurostrongylus falciformis* (Schlegel) of *Mustela vison* in Ontario.** ROY C. ANDERSON, The Ontario Research Foundation and AUDREY FYVIE, Ontario Department of Lands and Forests, Canada.

Aelurostrongylus falciformis was found in lungs of wild *Mustela vison* in Ontario. The following gastropods were infected experimentally: (a) the terrestrial forms *Zonitoides arborea, Discus cronkhitei, Deroceras gracile, Succinea ovalis,* and *Anguispira alternata;* (b) the quatic forms *Physa integra, Gyraulus deflexus, Armiger crista,* and *Ampullaria cuprina.* Mink-fed infected molluscs passed larvae 23 to 28 days later. Goldfish and mice served as transport hosts. Larvae were able to infect gastropods by penetrating the epidermis of the foot, but larvae ingested by slugs and snails resisted digestive processes, penetrated the gut wall, and developed successfully. Besides the foot, larvae developed to the infective stage in cysts on or within the body wall, on the wall of the gut, the surface of the liver, in the buccal mass, salivary gland and rarely the albumin gland. At 22 to 23 C the first moult occurred on the 5th to 6th day, the second on the thirteenth. There is a sigmoidal relationship between temperature and activity of first-stage larvae. Young *D. gracile* consumed larvae and became infected at 5 C. Desiccation killed larvae in a few minutes, and they survived freezing temperatures for only 4 or 5 days.

94. **The Influence of Temperature on Metabolic Response in Larval Trematodes.** WINONA B. VERNBERG, Duke University Marine Laboratory, Beaufort, N. C.

Two species of trematodes, *Zoogonus rübellus* and *Himasthla quissetensis,* utilize the same first intermediate host, *Nassarius obsoleta.* However, fish serve as the definitive host for *Z. rübellus,* while the adult stage of *H. quissetensis* is found in the herring gull, *Larus argentatus.* The metabolic rate of the sporocyst and cercarial stages of these two species of helminths was measured at intervals from 6 to 41 C. The response of the larvae to both the higher and lower temperature extremes can be closely correlated with the body temperature of their respective definitive hosts. Larva of *Z. rübellus* cannot withstand temperatures above 39 C, while the larvae of *H. quissetensis* tolerated 41 C for prolonged periods. Differences in response of the two species at lower temperatures are reflected in Q_{10} values.

95. **A Study of Spermatophores and Sperm Transfer in the Oribatei (Acarina).** H. G. SENGBUSCH, The State University of New York, Buffalo.

Relatively little information exists concerning fertilization in the oribatoid mites. Grandjean (1956) reported copulation in the Galumnidae, but his observations have not been confirmed. Pauly (1952, 1956) described the formation of spermatophores and sperm transfer in three species of Belbidae. Taberly (1957) substantiated the work of Pauly and listed 17 species in which he observed the production of spermatophores. Sengbusch (1958) added three more species to the above list.

The culture of oribatoid mites has been continued over many years. From innumerable observations, it is evident that copulation is not the usual method of sperm transfer employed. In all the non-parthenogenetic species studied, spermatophores were produced by the males and were subsequently picked up by the females. Each male is able to produce many spermatophores. The spermatophore consists of a slender pedicel which is anchored to the substrate by

means of an enlarged, sticky base. The free end bears a spherical capsule containing the spermatozoa. The female, with the genital covers open, positions herself over the spermatophore. The trifid ovipositor is extruded to clasp the sperm capsule and withdraw it into the body. Fecundation follows the breaking of the thin capsular membrane with the resultant release of spermatozoa into the vagina. It is suggested that the coupling behavior witnessed by Grandjean was not coital but possibly erotic.

The structure of spermatophores of six different species of oribatoid mites was studied. The results of this investigation will be discussed.

96. **On the Development and Function of New Accessory Glands of Ixodid Ticks Connected with Oviposition.** B. FELDMAN-MUHSAM AND Y. HAVIVI, The Hebrew University-Medical School, Department of Parasitology, Jerusalem, Israel.

Feldman-Muhsam and Havivi (Nature, 1960) reported on the presence of two large groups of glands in females of *Rhipicephalus*, *Hyalomma*, and *Boophilus*. The glands were not found in *Argasidae*. It could be shown that these glands start functioning with the onset of oviposition.

Further observations on the development of the glands during the pre-oviposition period and their presence in various genera of *Ixodidae* are reported in the present communication. Blocking of the outlet of the glands intercepted the flow of the secretion and showed that the secretion plays an indispensable part in the process of reproduction.

97. **The Identification of Larvae of the Genus *Argas* (Acarina: Argasidae).** D. E. SONENSHINE, C. M. CLIFFORD, AND G. M. KOHLS, University of Akron and Rocky Mountain Laboratory, Hamilton, Montana.

The study of larvae of 12 species in the genus *Argas* showed that chaetotaxy in conjunction with various other features affords useful diagnostic characters for subgenus and species identification.

Chaetotaxis features that had the greatest value were the number, arrangement and length of setae on the body, palp, and leg segments.

In addition to chaetotaxy, features such as the shape and dentition of the hypostome, length of the palpal articles, shape and length of tarsus I, as well as host preference and distribution, were found to provide useful recognition characters.

98. *Wohlfahrtia opaca* **Myiasis in an Adult Human Being.** ROBERT M. STABLER, Colorado College and Morgan Berthrong, Penrose Hospital, Colorado Springs, Colorado.

Myiasis due to the maggots of *Wohlfahrtia vigil* or *W. opaca* is not uncommon in the very young of dogs, cats, mink, rabbits, foxes, and man. The involvement of adult hosts, however, is virtually unknown. In man, parasitized infants are nearly all under 1 year, with most cases being less than 6 months of age. The 1 proven exception was that of a 4.5-year-old boy who had phimosis complicated by *W. vigil* larvae under his foreskin (O'Rourke, 1954, Can. Med. As. J. **71**: 146–149). The present case involving *W. opaca* myiasis in an adult human being is, therefore, of considerable interest.

The host was a 24-year-old electronics technician living in Colorado Springs, Colorado. In the latter part of August 1959 he noticed a raised, reddened nodule in the public hair just above the base of the penis. The nodule became quite tender. When the surface broke down he expressed a small "worm" by pressure. The object removed was a second-instar *W. opaca* maggot. Following his work, the man made a practice of taking a daily shower in the late afternoon and then lying sparsely clothed on his bed. He did not sun-bathe. He was aware of at least occasional flies in his room. It seems probable, therefore, that during one of his siestas in the semi-nude he was used as a host by a female *Wohlfahrtia opaca*.

99. **A Blood Parasite from Chickens in Wisconsin.** P. V. KIRSHNAMURTI, D. L. PEARDON, A. C. TODD, AND W. H. MCGIBBON, University of Wisconsin, Madison.

In the course of a survey of parasites in Wisconsin chickens, a parasite was observed inside erythrocytes in a blood smear from a laying pullet of the University flock of White Leghorns. The infection ranged from 1 to 3 parasites per 3,000 red blood corpuscles examined. When blood from such infected chickens was transfused to 3-week-old chickens, the initial parasitic forms appeared in erythrocytes in peripheral blood 8 to 12 days later. The parasites increased in number from the 10th day onwards till the 25th day, and different forms, apparently including trophozoites, schizonts, and gametocytes were observed. The infection has been maintained through a series of three groups of 3-week-old chickens.

The parasite is considered to be a *Plasmodium;* the species has not been established.

100. **A New Species of Chicken Coccidium.** S. A. EDGAR AND C. T. SIEBOLD, Auburn University, Alabama. (No abstract.)

101. **Intestinal Species of Coccidia in Chickens from Georgia.** W. M. REID, N. N. SHARMA, AND JOYCE KEENER, University of Georgia, Athens.

Studies have been continued on the degree of natural exposure to intestinal coccidia using the immunity challenge technique with 10 challenged and 10 non-challenged controls per flock. All flocks sampled have come from market age broilers between 7 and 10 weeks of age. All intestinal species thus far studied are very common throughout Georgia. The ranking by species in order of highest percentage of immune flocks is as follows: (1) the new species being described by Edgar, 100 percent immunity in 10 flocks; (2) *E. acervulina* 80 percent of 39 flocks; (3) *E. maxima* 58 percent of 43 flocks; (4) *E. necatrix* 52 percent of 68 flocks and (5) *E. brunetti* 17 percent of 69 flocks. There appears to be some correlation between the number of oocysts produced by these different species and the speed with which solid immunity is developed in different flocks in the field. In view of the large number of oocysts produced by Edgar's new species, the reasons for numerous outbreaks in laying flocks of Georgia and nearby states remain obscure.

102. **In Vivo Excystation of** *Eimeria acervulina.* MARION M. FARR AND DAVID J. DORAN, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

Eimeria acervulina oocysts, sporulated in potassium dichromate and freed from debris by sugar flotation, were inoculated into the crops of chickens 3, 13, and 44 days old. Examination of the birds 10 to 15 minutes, 25 to 30 minutes, and 1 hour after inoculation indicated that (1) the oocysts were apparently unchanged in the crop, (2) a high percentage of the oocysts were broken and their sporocysts released in the gizzard, (3) the sporozoites escaped from the liberated sporocysts in the duodenum and jejunum.

103. **In Vitro Excystation of** *Eimeria acervulina.* DAVID J. DORAN AND MARION M. FARR, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture.

All attempts to excyst sporozoites from intact oocysts with gastric and/or duodenal juice, pepsin, and various pancreatic enzyme preparations were unsuccessful.

Attempts to excyst sporozoites from free sporocysts mechanically released from oocysts using bile or pancreatic enzyme preparations produced low or insignificant percentages of excystation after 1 to 5 hours. In the presence of bile, bile salts, and other surface-active agents, the action of the pancreatic preparations was greatly increased. At pH 7.3 to 7.6, optimal concentrations of trypsin 2×, crystalline (salt free) and lipase (steapsin) in combination with 5 percent chicken bile produced 63 to 67 percent and 82 to 88 percent excystation, respectively. The heaviest suspension of motile, non-aggregating sporozoites was obtained with 0.25 percent (w/v) trypsin 1 to 300 in 5 percent chicken bile. Sometimes excystation was as high as 95 to 98 percent after 1 hour. Lipase (wheat germ), erepsin, and invertase were ineffective either in the presence or absence of bile. Optimal concentrations of sodium taurocholate and/or sodium glychocholate in combination with trypsin 1 to 300 produced percentages of excystation lower than with 5 percent bile and trypsin. Although sporozoites died shortly after emerging, 1.0×10^{-4} percent (v/v) Tween 80, 1.0×10^{-5} percent (v/v) tergitol, and 0.025 percent (w/v) Microsolv in combination with trypsin produced excystation as high as with 5 percent bile.

A comparison of percentages of excystation, inhibition of excystation with soybean trypsin inhibitor, and lipolytic and tryptic activities of the commercal pancreatic preparations indicates that an enzyme in addition to trypsin was responsible for excystation.

104. **The Activity of Chlortetracycline against** *Eimeria acervulina.* S. KANTOR AND R. L. KENNETT, JR., Agricultural Division, American Cyanamid Company, Princeton, New Jersey.

The prophylactic activity of chlortetracycline (the trademark of American Cyanamid Company for the antibiotic chlortetracycline is AUREOMYCIN) against severe *E. acervulina* infections in young chicks was studied in 3 diets with varied calcium content and source. Chlortetracycline eliminated or greatly reduced lesions in approximately one-third of the chicks at 440 to 500 ppm in both the basal diet containing no added calcium (0.4 percent Ca^{++}) and the diet containing 0.4 percent added calcium as calcium sulfate (0.8 percent Ca^{++} total). Weight gains did not differ significantly from those of the unmedicated, uninfected controls,

while unmedicated infected controls gained 30 percent less than the latter. In the 0.8 percent total Ca^{++} diet, lesions were eliminated from practically all the birds with 880 to 1000 ppm chlortetracycline. Less activity was obtained in a diet containing 1 percent added calcium as calcium carbonate (1.4 percent Ca^{++} total). In the latter, 1000 ppm of chlortetracycline prevented lesions in approximately one-half the chicks, though weight gains comparable to uninfected unmedicated controls were attained with lower concentrations.

105. **Zoalene Tolerance by** *Eimeria tenella.* D. K. McLoughlin and J. L. Gardiner, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.

A strain of *Eimeria tenella* that heretofore had never been exposed to drugs was serially propagated in groups of chickens that initially were fed suboptimal levels of zoalene. The birds of passages 12 through 17 were fed the chemical at the usual level of 0.0125 percent. The experimental strain developed a tolerance for zoalene as evidenced by a depressed rate of gain, increased severity of cecal lesions, failure of the chemical to suppress oocyst production and, finally, death from coccidiosis. Control lots of oocysts passed an equal number of times through unmedicated birds were fully susceptible to zoalene. This indicates that the inability of the drug to afford protection to chickens inoculated with the experimental strain of coccidia was due to drug tolerance rather than to increased virulence. Oocysts recovered from medicated and unmedicated birds, respectively, of passage 17, were given to groups of birds fed mash containing nicarbazin, Trithiadol, Unistat, glycarbylamide, arsenosobenzene and nitrofurazone. Only with nitrofurazone was there any indication of cross-resistance by the zoalene-tolerant strain.

106. **Ultrastructure of the Cuticular Region and Flame-Cell System of the Metacercaria,** *Diplostomulum trituri.* E. W. Lautenschlager, University of Virginia, and R. R. Cardell, Jr., Edsel Ford Institute for Medical Research, Charlottesville.

Diplostomulum trituri, existed as an active, unencysted parasite of the brain case of the newt, is of particular interest for the study of the ultrastructure of those regions which may be associated with the absorptive and excretory functions in the metacercarial stage of trematodes.

As previously reported (Lautenschlager and Cardell, 1959), the cuticle appears as a rather homogenous layer containing many small vacuoles which may be preparation artifacts. There is a distinct basement membrane separating the cuticle and the underlying muscle layers. Although not clearly established, there is some indication that channels may exist through the muscle layers from the cuticle to the sub-muscular region.

The flame-cells of this metacercaria follow the structural pattern previously reported for other trematodes by Kümmel (1958), Senft, Philpott and Pelofsky (1961), and others. The excretory tubules of this form display distinct filaments projecting from the wall of the tubule similar to the structure reported by others for the adult *Fasciola hepatica* and *Schistosoma mansoni* and the cercaria of *Himasthla quissetensis.* The similarity of structure of the protonephridial systems of these different trematode groups and stages of development may establish this as typical structure of the trematode protonephridial system and permit more definite understanding of the function of the system.

107. **Chemotactic Responses of Snails Infected with** *Schistosoma mansoni.* Frank J. Etges, University of Cincinnati.

Studies by Michaelson (1960) and Etges (1960), utilizing different methods, indicate that uninfected schistosome vectors, *Taphius* (Syn. *Australorbis*) *glabratus* and *Bulinus truncatus,* are stimulated and attracted by several crude vegetable materials (viz, lettuce, banana, water hyacinth, and wheat germ). The chemical basis for chemo-attractive substances remains undefined.

Current studies, using snails experimentally infected with a Puerto Rican strain of *Schistosoma mansoni* at various stage of development, reveal that the degree of response to a given attractant gradually lessens as the infection develops, but never ceases until the snail's death. Comparisons of responses among control and experimental groups indicate that decreased movement toward attractants by infected snails is due to impaired chemosensitivity rather than decreased mobility. Snails with infections of long standing, 4 months or more, from which large numbers of cercariae emerged daily, were as active as uninfected snails, but their movements were random in the presence of attractive substances. Since the parasites are not found in close proximity to the osphradia, their ganglia or nerves, an indirect mechanism of inhibition of chemoreception is suggested.

108. **Host Hibernation and Its Effects on *Schistosomatium douthitti* and *Schistosoma mansoni* (Trematoda: Schistosomatidae).** D. W. DERY, The College of Charleston, S. C.

Five separate experiments were conducted, designed to furnish data concerning the effects of host acclimatization and/or hibernation upon: infectability, survival, development, and egg production of schistosomes. Hosts were exposed to schistosome cercariae (*S. douthitti* or *S. mansoni*) either before or after cold acclimatization; a randomly selected group was put into a cold room to hibernate; others served as controls at room temperatures. After various lengths of time the hosts were autopsied in an attempt to recover all schistosomes.

The 72 hosts yielded a total of 3,399 schistosomes, of which 3,069 were stained, mounted, and examined. Of the total, 1,721 were recovered from refrigerated and 1,678 from control hosts.

Experimental hosts hibernated for periods ranging from 1 to 58 days with continuous periods from one-half to 14 days.

The following conclusions were reached: (1) Ground squirrels served as hosts for *S. douthitti*, but were more resistant than mice; (2) cold acclimatized hosts were infected; (3) schistosomes survived cold acclimatization of the hosts; (4) schistosomes developed and produced viable eggs in cold acclimatized hosts; (5) some adults survived host hibernation without apparent abnormality; (6) development during host hibernation was retarded; (7) eggs were produced in hibernating hosts and miracidia developed and hatched; and (8) visceral temperatures of hamsters in deep hibernation were essentially that of the cold room environment.

109. **Schistosomule vs Cercaria.** M. A. STIREWALT, Naval Medical Research Institute, Bethesda, Md.

Cercariae of *Schistosoma mansoni* have been compared with schistosomules recovered after 15 minutes, 1 hour, 2 days, 4 days and 7 days in previously uninfected and in previously infected mouse skin. Important differences have been noted. Among them are the following: Cercariae are water-adapted while water is cidal to schistosomules; to cercariae, serum and physiological salt solution are toxic, but schistosomules are serum and saline adapted. No pericercarial envelopes were found around any larvae recovered from skin. Furthermore, schistosomules did not form envelopes in vitro in serum from infected mice even after so short a period as 15 minutes in skin.

110. **Some Factors Influencing Penetration of *Schistosoma mansoni* Cercariae in Mice Exposed to Infested Flowing Waters.** MYRON G. RADKE, L. S. RITCHIE, AND WILLIAM B. ROWAN, Department of Medical Zoology, Walter Reed Army Institute of Research, Washington 12, D. C. WRAIR Field Team and USPHS, San Juan, Puerto Rico.

Laboratory and field investigations were undertaken to define the viability of *Schistosoma mansoni* cercariae in relation to water velocity and distance from point of release. Mice were exposed for various intervals of time to known numbers of *S. mansoni* released into flowing water.

The number of adult worms recovered in mice decreased as the water velocity increased. Animals exposed to cercariae transported at 2.0 and 2.7 miles per hour yielded an average schistosome burden of 106 and 43, respectively. Mice exposed intermittently to cercaria-infested water had comparable worm burdens to those exposed continuously for the same length of time. The cercariae were not prevented from penetrating by washing the mice with cercaria-free flowing water.

No loss in the number of released cercariae carried downstream up to 2000 feet was detected with Rowan's cercariometer. The worm burden of animals placed in cercaria-infested flowing water was inversely proportional to the distance from the point of cercarial release. The worm burden which animals acquired from cercaria-infested water increased with longer exposure intervals. However, the calculated cercarial infectivity rate was considerably higher for shorter exposure intervals of 1 and 5 minutes.

111. **Relationship of Antibody Nitrogen to Titer Obtained in the Cercarial Antigen Slide Flocculation Test for Schistosomiasis.** R. I. ANDERSON, Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D. C.

The amount of antibody protein required to produce a minimal positive reaction in the cercarial antigen slide flocculation test was studied in sera from patients with *Schistosoma mansoni* infections. Sera with various titers in the flocculation test were selected and absorbed with the cercarial antigen flocules. The absorption from each serum of antibody producing a demonstrable reaction in the test was accomplished with a single calculated volume of

washed packed antigen floccules. Analysis for absorbed antibody nitrogen performed on the initial, and a repeated, absorption showed that essentially all of the antibody nitrogen was also removed by the single calculated volume of antigen. Absorption of sera obtained from uninfected individuals yielded no detectable nitrogen. When the amount of antibody nitrogen obtained from sera with various titers was plotted on arithmetic ordinates against the reciprocal of the serum dilution (titer), a straight line was formed. It was estimated that 0.27 mg of antibody protein per ml of serum was required to give a minimal positive slide flocculation test.

112. **Susceptibility of Mammalian Hosts to Experimental Infection with** *Heterobilharzia americana*. H. F. LEE, Tulane University School of Medicine, New Orleans.

Observations were made on the susceptibility of the albino mouse, hamster, dog, raccoon, cotton rat, rabbit, guinea pig, albino rat, and rhesus monkey to infection with *Heterobilharzia americana*. The schistosome was maintained in mice and dogs, and in laboratory-reared *Pseudosuccinea columella*. Criteria of suspectibility were: (a) Percentage of cercariae recovered as worms; (b) degree of maturation of the parasites; (c) production of viable eggs; and (d) prepatent period of the infection.

Cercariae were observed to penetrate the skin of all hosts tested, but there was great variation in the degree of development in the various hosts. In the mouse, hamster, dog, raccoon, and cotton rat the worms attained optimal development and discharged viable eggs in the feces; 32 to 73 percent of the administered cercariae were recovered as adults, and eggs appeared in the feces 64 to 87 days after exposure; the death rate among the infected hamsters was high and marked clinical manifestations developed in the dog. The cat, rabbit and guinea pig exhibited a degree of host resistance which did not bar maturation of the worms but did affect the production of viable eggs; 36 to 58 percent of the administered cercariae were recovered as adults but some of them remained immature and none produced normal eggs. The albino rat and monkey were very resistant; only 2 percent of the cercariae were recovered as stunted worms in the rat and none could be found in the monkey (three exposed and examined for eggs; one examined for worms).

113. **Resistance Induced in Mice and in Monkeys with the Japanese and Formosan Strains of** *Schistomosa japonicum*. E. H. SADUN, A. YAMAKI, S. S. LIN, AND J. C. BURKE, Departments of Medical Zoology, Walter Reed Army Institute of Research, Washington, D. C., and 406th Medical General Laboratory, Tokyo, Japan.

Superinfection with the Formosan and Japanese strains of *S. japonicum* in mice infected with the homologous and heterologous strains was characterized by a significant reduction in the number and length of developing worms from the challenging infection. When the challenging dose was given 10 days after the initial infection significant resistance was observed only among animals immunized with the Formosan strain. When the challenge was given 30 days after exposure a marked reduction in the worm burden was observed in all groups. The greatest reduction in the number and length of worms of the second infection was observed when the challenge was given 60 days after exposure. This reduction was even greater than that observed when the animals were challenged 90 days after the first exposure.

In general, the greater and more rapidly manifested degree of resistance was observed in mice which had been initially infected with the Formosan strain. No significant effect was detected in worms of the primary infection resulting from superinfection with the homologous or heterologous strain. No significant effect resulting from superinfection with the homologous or heterologous strain was detected in the worms of the primary infection. Results in Rhesus monkeys confirmed observations made with mice.

In addition to demonstrating that a cross protection is possible in mice and monkeys with the Formosan and Japanese strains, these experiments offer evidence in support of the view that the presence of schistosome eggs in the host's tissues is not necessary for the development of acquired resistance.

114. **Natural and Experimental Infections of Snails and Mammals with** *Schistosoma bovis*. EMILE A. MALEK, Tulane University School of Medicine, New Orleans.

Data not previously reported have been collected on the snail and mammalian hosts naturally infected with *Schistosoma bovis* in the Sudan. The infection was found in *Bulinus* (*Physopsis*) *ugandae* and *Bulinus* (*Bulinus*) *truncatus*, and in cattle, sheep, goats, horses, donkeys, camels and pigs. The main foci of the infection are in the following provinces: White Nile, Bahr-el-Ghazal, Kordofan, and Darfur. Experimental infections were established in white mice, guinea pigs, rabbits, a monkey (*Cercopithecus aethiops*), and in various wild mammals

including the Nile rat (*Arvicanthis niloticus*), gerbil (*Gerbillus gerbillus*), spiny mouse (*Acomys cahirinus*), and hedgehog (*Paraechinus aethiopicus*). Worms in these hosts were located in branches of the intrahepatic portal vein and mesenteric venules along the entire length of the large and small intestines. Experimental infection in the various hosts differed as to the percentage of cercariae recovered as worms, size of the worms, number of eggs in the worm's uterus, and abundance of eggs in the tissues and feces. The pathologenicity of *S. bovis* infection is fundamentally similar to that of several other species of schistosomes. The best hosts for laboratory maintenance of *S. bovis*, in descending order, are: Nile rat, white mouse, spiny mouse, rabbit, and guinea pig. There was a high percentage of development of worms in these hosts (35 to 52 percent of the cercariae administered) and an abundance of eggs in the liver, intestinal wall and feces after 64 to 78 days of infection. In the monkey worms developed to maturity but deposited few eggs in the liver and intestinal wall and none were observed in the feces. In experimentally infected natural hosts (sheep, goat, calf, and donkey) the prepatent period averaged 75 days.

115. **The Histopathology of Schistosomiasis in Abnormal Hosts.** F. VON LICHTENBERG, E. H. SADUN, AND J. I. BRUCE, Department of Pathology, Peter Bent Brigham Hospital, Boston, Massachusetts, and Department of Medical Zoology, Walter Reed Army Institute of Research, Washington, D. C.

Detailed histopathological examinations of the tissues of hosts with varying degrees of resistance to *S. mansoni* were made in the hope of observing differences that might give some clues to the mechanisms involved in abnormal hosts.

Examination of skin, lung, liver, and intestine from 13 species of normal and infected animals confirmed the wide spectrum of host parasite relationship outlined by parasitological methods. However, a review of the data in the light of added dimensions of tissue pathology emphasizes that parasitological observations are the result of a balance among several factors, some of which may be antithetical to others. The Hoeppli phenomenon was observed in the woodchuck. In the opossum eggs for the most part failed to gain access to the intestinal mucosa and the organ loads were very high. In the cottontail rabbit, the liver was the sole target and it absorbed nearly the entire egg production of the relatively few adults. In the muskrat parasite development was stopped before adulthood was attained, but schistosomula reached the lungs where some evoked a granulomatous response and were trapped. In the fox, schistosomula were trapped in the corion and subcutaneous tissue and were destroyed in situ within a week of penetration. Throughout this series a good correlation was observed between the number of eggs in the tissues, the degree of host reactivity to eggs expressed by size and cell composition of granulomas and the severity of organ damage in the host. As egg deposition increased, granulomas tended to become larger, more florid, cellular, and organ impairment increased. The striking similarity between the cellular reactions observed in the abnormal hosts and those in immunized laboratory animals constitutes a vital biological phenomenon of considerable interest.

116. **Some Factors Affecting "Immunity" to *Schistosoma mansoni* Infections in Mice and the Possible Immunogenic Role of the Schistosome Egg.** RICHARD B. CRANDALL AND GEORGE W. HUNTER III, University of Florida, Gainesville.

Variables in experimental design which might influence the development of acquired immunity to *Schistosoma mansoni* in mice, induced by previous cercarial exposures to the homologous species, were investigated. The variables considered were: (1) The number of "immunizing" cercarial exposures to which the mice were subjected; (2) the time interval between the "immunization" and challenge exposures; and (3) the size of the challenge exposure. The statistically significant results indicate that the number of "immunizing" exposures and the size of the challenge exposure were not important, but that a period of approximately 60 days from the initial cercarial exposure was required before increased resistance to a challenge infection was apparent. These results suggest that immunity to superinfection may only be demonstrable after the initial "immunizing" infection had matured and marked egg deposition had occurred. In addition evidence is found in preliminary experiments that injections of living *S. mansoni* eggs may increase resistance (stastistically significant) to an homologous challenge infection in mice.

117. **Biological Potentials of *Australorbis glabratus* (Puerto Rico): Growth Rate, Age at Onset of Egg-Laying, Egg Production and Life Span.** L. S. RITCHIE, L. A. BERRIOS-DURAN, R. DEWEESE, AND R. ROSA-AMADOR, U. S. Army Tropical Research Medical Laboratory, Fort Brooke, San Juan, Puerto Rico.

Observations indicate that biological potentials of growth and reproduction of *Australorbis glabratus* have been approximated in our investigations. This is deemed important for (1) laboratory and field studies involving this snail, (2) comprehending the epidemiology of bilharziasis, and (3) expediting chemical control of the snail. Static, circulating and continuously flowing water under laboratory conditions have been used. Onset of egg-laying has been obtained repeatedly within 21 days from hatching, with a majority of the snails laying within 2 days thereafter. Growth to a mean diameter of 20 mm has been obtained in 60 days in one experiment. Two clutches (40 to 50 eggs each) per snail day have been produced for as long as 6 weeks by 20 snails in slowly flowing water. The potential propagative period, total egg production and life span remain uncertain.

118. **Quantity and Infectivity of *Schistosoma mansoni* Cercariae Emerging from *Australorbis glabratus* Maintained on Alginated Food.** D. G. ERICKSON AND D. R. CALDWELL, U. S. Army Tropical Research Medical Laboratory, Fort Brooke, San Juan, Puerto Rico.

Studies at this laboratory (Erickson et al, 1961, J. Parasit. (Supp.)) have shown that *Australorbis glabratus* fed only food containing sodium alginate exhibit a slower growth rate and decreased egg production as compared with snails fed the same food without alginate. Further experiments were conducted to study possible effects of alginated food on emergence and infectivity of *Schistosoma mansoni*.

Cercarial production of snails raised and maintained on alginated food prepared as described by Moore et al, (1953, J. Parasit. **39**: 215–221) was compared with that of snails fed the same food formula without the alginate or the latter food together with leaf lettuce. All snails were raised from a single progenitor, handled identically except for food, and were exposed to the same hatching of miracidia. Determinations of cercarial production were begun at 6 weeks after exposure and were made 1 day a week for 9 weeks.

Snails fed alginated food discharged significantly fewer cercariae than the control snails. Mean daily cercarial production per snail in one 9-week trial was 7150 ± 2296 (S.D.) for the control and 3262 ± 616 for the snails fed alginated food. Comparable results were obtained in three trials. Control snails placed on the alginate diet after 4 weeks of shedding discharged significantly fewer cercariae in the next 5 weeks than those continued on the original non-alginated diet. Alginate-fed snails placed on the control diet at this same time discharged significantly more cercariae in the next 5 weeks than those maintained continuously on the alginated food.

There was no significant difference in worm burdens in mice exposed to cercariae from snails fed alginated food or cercariae from snails fed the control diet.

119. **Growth and Reproduction of *Australorbis glabratus* Fed Alginated Food.** D. G. ERICKSON, L. S. RITCHIE, AND D. R. CALDWELL, U. S. Army Tropical Research Medical Laboratory, Fort Brooke, San Juan, Puerto Rico.

The alginated snail food described by Standen (1951, Ann. Trop. Med. Parasit. **45**: 80–83) and modified by Moore et al, (1953, J. Parasit. **39**: 215–221) was evaluated on the criteria of growth rate and egg production of *Australorbis glabratus*.

Snails fed only alginated food prepared as described by Moore et al, (1953) were compared with control snails fed the same formula of food ingredients without the alginate or with snails fed the latter food and leaf lettuce. Snails fed food containing any 1 of 5 different grades, or brands of sodium alginate, either jelled into sheets with $CaCl_2$ or mixed with the food formula and fed as a powder or paste, showed highly significant reduction of growth rate. Considerable difference was noted in this effect with different grades of alginate. Significantly decreased egg production occurred with certain of these alginates also. The shells of snails raised on alginated food were thin and fragile as compared with those of the control snails. Snails fed the food formula mixed with talc in place of alginate in a quantity equal to that in alginated food showed a growth rate and egg production comparable to that of control snails fed the food formula alone. The growth rate and egg production of these talc-fed snails was significantly greater than that of snails fed alginated food. Further experiments of this nature established that the bulk of alginate in alginated food is not in itself the cause of this inhibition of growth and egg production.

Normal growth rate and egg production was attained 3 to 4 weeks after alginate-raised snails were placed on the control diet.

120. **Exposure of South Florida Snails of the Genus *Tropicorbis* to Miracidia of *Schistosoma mansoni*.** W. HENRY LEIGH, University of Miami.

Drainage canals in the farmlands of southwest Dade County, Florida, are heavily popu-

lated with a planorbid snail tentatively identified by Dr. C. S. Richards as *Tropicorbis obstructus*. Several species of the genus *Tropicorbis* are known to serve as natural vectors of *Schistosoma mansoni*, or have been experimentally infected with the schistosome. Since the farm lands of South Florida attract a large number of migrant workers from Puerto Rico, an endemic area for schistosomiasis, it was desirable to ascertain the susceptibility of this snail to *S. mansoni*.

Many thousands of these snails were collected but the only cercariae released belonged to an unidentified species of echinostome. Many snails were also infected with unidentified metacercariae. Both field collected and laboratory reared snails were exposed to miracidia of *S. mansoni* taken from mouse livers. Snails, varying from 1 to 4 mm in diameter, were isolated in small iodine dishes to achieve maximum contact of snail and miracidia and exposed to miracidia varying in number from 10 to 50. The majority of snails exposed were reared in the laboratory.

Over a period of almost 1 year 1109 snails were individually exposed and periodically checked for emergence of cercariae. Observations were discontinued after a period of 3 to 4 months following exposure. Only 243 snails lived longer than 3 months. While miracidia appeared to penetrate the snails, none of the snails produced cercariae and no sporocyst stages were found. Snails heavily exposed to miracidia, killed and fixed for sectioning, have not yet been examined for the presence of developing sporocysts. This study will be continued using younger snails and multiple exposures to miracidia. (This study supported by Grant E-223 of the National Institutes of Health.)

121. **The Growth Difference of** *Oncomelania nosophora* **and** *O. hupensis*, **the Vector Snails of** *Schistosoma japonicum*, **Between Sexes.** Y. KOMIYA AND K. KOJIMA, Department of Parasitology, National Institute of Health, Tokyo.

Parent snails of both species were reared in the laboratory. The newly hatched snails were about 0.3 mm in shell length. It took about 1 month for them to attain about 2 mm in shell length, at which time they were transfered to a Petri dish of 10 cm diameter for rearing. Their shell length was measured every month. When they attained about 6 mm in shell length, they were sexed.

The results show that the growth rate of the male in both species was larger as compared with that of the female. Its difference became much larger when snails reached up to 5 mm or more in shell length. The ratio of the shell length of the male to female was 1.6 in *O. hupensis* whereas in *O. nosophora* it was 1.16 when they attained to about 6 mm in shell length.

122. **Follow-Up Studies of Chemotherapy of Paragonimiasis with Bithionol.** M. YOKOGAWA, H. YOSHIMURA, T. OKURA, M. SANO, M. TSUJI, M. IWASAKI, AND M. SHIGEYASU, The Chiba University, Japan.

It was found by the authors that Bithionol (2.2-Thiobis, 4-6-dichlorophenol) had a remarkable potency for killing *Paragonimus westermani* in vitro as well as in animal experiments. The authors applied this drug for the first time to the treatment of human paragonimiasis. A total of 13 cases were given Bithionol in the daily dose of 50 mg per kg (in 3 divided "tekes") and every other day in three subgroups, one receiving 15 doses (8 cases), one 10 doses (4 cases), and one 5 doses (1 case).

The follow-up studies for 6 months after treatment have been completed. No relapses were found. It was concluded that all cases were completely cleared with Bithionol. The Chest-X-Ray findings of those treated patients taken before and at monthly intervals after treatment were compared. Chest-X-Ray findings of paragonimiasis (nodular-, cystic-, infiltrative-, bronchiectatic-types and increased) lung markings were found before treatment. Most of these shadows diminished in size or disappeared within 1 to 5 months after treatment.

Complement-fixation tests were performed on these patients before and monthly after treatment. In 3 cases positive reactions in C-F test became negative one month after treatment and in 4 cases the antibody titers in C-F Test dropped to less than $\frac{1}{2} - \frac{1}{3}$ of those before treatment within 3 to 6 months after treatment. No decrease of antibody titers was found in the other two cases during the period of 6 months after treatment.

Comparisons of the hemogram and serum proteins before and after treatment were also made.

123. **The Newest Lung Fluke in Japan,** *Paragonimus miyazakii* **Kamo, Nishida, Hatsushika and Tomimura, 1961.** I. MIYAZAKI, Kyushu University, and H. KAMO, Tottori University, Japan.

In 1955, Miyazaki found a species of *Paragonimus* from some weasels in Kyushu, which he regarded as *P. kellicotti* after a detailed comparison with American specimens of the same

species. Since then, the same worm has been obtained from weasels and yellow martens in Kyushu and Honshu as well as from a dog in Honshu. Kamo et al (1961, Yonago Acta Med. 5) found a new metacercaria from a crab *Potamon* (*Geothelphusa*) *dehaani* in Yamaguchi Prefecture, Honshu, and experimentally obtained adult worms from a cat and an albino rat, which were fed with this metacercaria. These adults were proved to be the same as the worms previously found in weasels, martens, and a dog. They named this species *P. miyazakii*, showing some fundamental differences from *P. kellicotti*.

Of 171 crabs examined, 37 or 21.6 percent were found to harbor 301 metacercariae in all, of which 202 or 67 percent were parasitic in the muscle and 99 in their liver. No larva was found in the cardiac region of the crab. The metacercaria is enveloped in double cysts: The inner one is usually spherical in shape and measures 426 to 515 microns, averaging 473.8 microns in diameter; its wall is strong, measuring 10.3 to 27.3 (averaging 19.5) microns in thickness. The outer cyst is a little larger, but its wall is somewhat thinner, measuring 15.9 microns on the average. As compared with *P. kellicotti*, the metacercaria of the present species is a little larger in diameter and the wall of outer cyst seems to be thicker, but that of inner cyst thinner.

Cuticular spines of the adult worm are mostly singly spaced and its ovary is delicately branched. These features are quite similar to *P. kellicotti*, but eggs of the present worm are smaller (73.4 × 43.8 microns on the average) and their shell is generally thinner (1 to 2 microns).

From the medical point of view, it is significant that the metacercariae of *P. miyazakii* are parasitic in *Potamon dehaani*, which is the well-known crab host of *P. westermani* and is sometimes eaten uncooked. On the other hand, the existence of *P. kellicotti* in Japan, which was reported at the 34th Annual Meeting by Miyazaki (1959, J. Parasit. **45** (Supp.), became very doubtful.

124. **The Effect of the Larvae of *Glypthelmins pennsylvaniensis* (Trematoda: Brachycoeliidae) on Glycogen Deposition in the Hepatopancreas of *Helisoma trivolvis* (Say).** RANDALL W. SNYDER, JR. AND THOMAS C. CHENG, Lafayette College, Easton, Pa.

Earlier investigations (Cheng, T. C., J. Parasit. In press) revealed that the snail *Helisoma trivolvis* serves as the only intermediate host for the trematode *Glypthelmins pennsylvaniensis* Cheng, 1961. The daughter sporocysts and escaping cercariae are found within the hepatopancreas of the mollusc. This phase of the investigation was conducted to determine the effects of the parasite on the snail host. Glycogen deposition in the snail was studied in infected and uninfected specimens by utilizing the PAS-Schiff reaction. In both instances diastase-digestion was used as control. It was determined that in the infected snails the glycogen within the hepatopancreatic cells is greatly reduced, visible only as fine granules adhering to the inner surfaces of the cell membranes. Even these granules are not apparent in diastase-treated sections. In uninfected snails the hepatopancreatic cells are filled with glycogen. The control series revealed the substance to be glycogen since it was readily digested. It is postulated that the depletion of glycogen in the host's hepatopancreas has resulted from its being utilized by the parasites since appreciable amounts of glycogen were observed within the latter. (This work was supported, in part, by Research Grant E-3443 from the Division of Allergy and Infectious Diseases, National Institutes of Health.)

125. **Experimental Inversion of the Primary Axis in the Developing Egg of *Spirometra mansonoides*.** JUSTUS F. MUELLER, S.U.N.Y. Upstate Medical Center, Syracuse 10, New York.

With the exception of a single figure by Vergeer (1935) most authors who have worked with the developing egg of diphyllobothriid cestodes imply, or expressly state, that the oncosphere forms with the hooks directly away from the operculum. Since the hooks mark the posterior pole of the oncosphere, the coracidium is thus lined up in the most favorable position for hatching, with the anterior end pointed toward the escape opening. In the last few days before hatching the embryo performs sluggish contractions, but these do not appear adequate to effect any change in the orientation of the oncosphere within the egg.

When eggs are incubated at 37 C there ensues a heavy loss so that a much smaller percentage of eggs than normal develop. It has been discovered recently that many of these eggs that develop under heat stress show very incomplete utilization of yolk cells, with small coracidia; and a proportion of coracidia lie in the inverted position, with hooks directed toward the operculum. Many of these inverted embryos are large and appear to have developed in that position, rather than to have turned around inside the shell. Susceptibility to this temperature effect is enhanced in eggs which have been stored in the cold for 2 years or more. In some lots of eggs, held at 37 C throughout incubation, as many as 20 percent of the embryos

are inverted. When eggs are removed from the 37 C incubator at 3, 3.5, or 4.5 days, before any trace of hooks has appeared, and continued at room temperature, a smaller proportion show inversion, from 2 percent to 5 percent respectively. In each case an additional number of eggs show transverse or angular orientation of the contained larvae, but always fewer than show complete inversion.

The position of these inverted larvae appears to present an obstacle to hatching, which may account for a gradual increase in their number in older cultures of eggs. But whether they hatch or not has not been determined. In addition to such inverted embryos and miniature larvae, other highly abnormal larvae are found in such cultures reared under stress. These include forms in which hooks are completely lacking although the larvae is ready to hatch, forms in which flame cells appear external to the finished coracidium, or eggs in which ciliated epithelium, hooks, and flame cells are all present, but in bizarre arrangements. Obviously the tapeworm egg has a mosaic type of development. (Supported by continuing grants from the National Institutes of Health, E-1876.)

126. **Morphological Develoment and Maturation of *Hymenolepis diminuta* in the Definitive Host as Affected by Population Density.** L. S. ROBERTS, The Johns Hopkins University, School of Hygiene and Public Health, and the Institute of Parasitology, Macdonald College of McGill University, Canada.

The characteristics of growth in length and weight of *H. diminuta* in infections of varying intensity have been reported earlier (Roberts, 1960, J. Parasit. **46** (Supp.): 11). Daily samples of worms from population sizes ranging from 1 to 100 worms per host were fixed in alcohol-formalin-acetic fixative, stained with Semichon's acetic carmine, and mounted on glass slides. Observations on morphology and proglottid number were performed. The rate of maturation and the length of the prepatant period were essentially independent of population density, rate of growth in length and weight, and the rate of proglottisation. Determination of a time scale of maturation showed the following characteristics in all populations: (1) 4 days postinfection, beginning of proglottisation with formation of the genital anlage; (2) 6 to 7 days, maturation of the terminal proglottids; (3) 9 to 10 days, developing ova in the uteri; (4) 12 to 13 days, formation of the earliest gravid proglottids as judged by the presence of shelled eggs with well-developed oncospheres, and (5) 16 to 17 days, patency. The number of immature proglottids was unaffected by population size, the mature proglottids were reduced in number by high population density, and the gravid proglottids were most severely affected. (This investigation was supported in part by a research grant, E-1508, from the National Institutes of Health, United States Public Health Service, and it was carried out during the tenure of a Predoctoral Fellowship from the United States Public Health Service.)

127. **The Influence of Population Density on Changes in Chemical Composition of *Hymenolepis diminuta* During Its Growth in the Rat.** L. S. ROBERTS, The Johns Hopkins University, School of Hygiene and Public Health, and the Institute of Parasitology, Macdonald College of McGill University, Canada.

Total carbohydrate, nitrogen, lipid, and lipid phosphorus were determined in samples of worms from infections of varying intensity. The effects on increase per worm of these components by population density were similar to those noted for length and fresh weight (Roberts, 1960, J. Parasit. **46** (Supp.): 11). The effect of population was greatest on the amount of carbohydrate per worm, and it was least on lipid. Nitrogen determinations on artificially excysted worms showed the initial value to be 6.11×10^{-3} micrograms per worm. The carbohydrate percentage of dry weight increased from about 20 percent at 4 days to 40 percent at 17 days in worms from small populations, but little or no increase was noted in larger populations. The smaller percentage of carbohydrate in worms from crowded infections was partially made up by an increased percentage of lipids. The percent protein fell from 60 to 70 percent to about 30 percent, and the phospholipid fell from 10 percent to 3 to 4 percent in all populations. Calculation of the heterauxetic rate constants (see Needham, 1950, Biochemistry and Morphogenesis) with relation to increase in dry weight showed that protein and phospholipid were bradyauxetic, carbohydrate was tachyauxetic and had a tendency toward isoauxesis with increasing population size, and lipids were bradyauxetic or isoauxetic with a tendency toward tachyauxesis in higher populations. Although the data indicate that carbohydrate plays a role in the crowding effect, the direct cause is still in question. (This investigation was supported in part by a research grant, E-1508, from the National Institutes of Health, United States Public Health Service, and it was carried out during the tenure of a Predoctoral Fellowship from the United States Public Health Service.)

128. **In Vivo Antibody Binding Sites in *Hymenolepis nana* as Demonstrated by Direct and Indirect Immunofluorescent Staining.** R. M. COLEMAN, Russell Sage College, Troy, New York.

Anti-*Hymenolepis* rabbit globulins were prepared and conjugated with Lissamine Rhodamine B 200 as previously described (Coleman and Dutt, 1960, J. Parasit. **46** (Supp.): 12) whereas the fluorescein isothiocyanate conjugates were prepared essentially after Coons' method. Each of the experimental mice utilized weighed approximately 22 g. when orally infected with 5000 eggs. Each mouse from group A, at the end of a 2-week period of infection, received 0.2 ml of an anti-*Hymenolepis* rabbit globulin, conjugated with rhodamine. The conjugate was introduced, via the tail vein, on each of 4 successive days whereupon the mice were autopsied. The same procedure was followed for mice of group B 3 weeks after they had received the eggs. Tapeworms from groups C and D, following 2- and 3-week periods of infection, respectively, were stained with an anti-mouse globulin conjugated with fluorescein isothiocyanate. Some worms from all groups were stained directly with rhodamine conjugates. Controls involved: (1) Heat inactivation of sera; (2) treatment of conjugates with liver powders (mouse and beef); (3) treatment of conjugates with charcoal; (4) treatment of worms with uncoupled normal and immune sera (rabbit and mouse); (5) staining with dyes not coupled with protein; and (6) staining with normal sera conjugated with rhodamine. Fluorescent microscopy was accomplished by the use of a Reichert-Hacker assembly and microscope with appropriate filters.

In vivo antibody binding sites will be discussed and demonstrated. Sites were partially occupied with mouse immune globulin at the end of a 2-week period of infection and almost entirely occupied at the end of the third week. (This investigation was supported, in part, by a research grant, G 14342, from the National Science Foundation and by grants from the Sigma Xi-Resa Research Fund and Russell Sage College.)

129. **Somatic Pairing of Chromosomes in Normal and Mutant Strains of *Hymenolepis diminuta*.** LEE T. DOUGLAS, The Johns Hopkins University, Baltimore.

During cleavage of *H. diminuta* the number of visible chromosomal units is reduced from 12 to 6. The hypothesis most consistent with the data is that this process is accomplished by somatic pairing of homologous chromosomes. Pairing appears to be quite intimate, but unequal distribution of anaphase pairs suggests that homologs sometimes separate from each other in metaphase or early anaphase. Alternative hypotheses to explain reduction in chromosome number do not appear tenable for the following two reasons: (1) Neither chromosomal diminution nor extrusion have been observed in over 4000 embryos studied and (2) all possible stages of pairing are seen in division figures (pairing seems to begin at one end of the homologs and proceed in "zipper" fashion to the other end). The importance of somatic pairing to survival of the species is evident provided it enhances somatic crossing-over. Somatic crossing-over in germinal tissue near the scolex could provide for phenotypic variation among proglottids.

In mutant material (9th and 10th generation offspring of an X-irradiated scolex) the types of pairing figures observed suggest that translocations (and possibly inversions) were produced in parental stock, underwent genetic fixation by inbreeding and are presently being maintained. An example is chromosome 1 (idiogram number) of mutant material. During prometaphase it is often seen with a terminal oval loop—one side of the loop pairing (if at all) with number 2 and the other side with chromosome number 3. The loop may be formed independently of the pairing. The occurrence of the loop together with the observed pairings suggests that chromosome 1 in mutant worms is actually composed of the following segments: (1) number 1 proper, (2) translocated fragments of numbers 2 and 3, and (3) a terminal piece from the homolog of number 1. Preliminary attempts to correlate specific chromosomal aberrations with anomalies in proglottid morphology have not proven fruitful.

130. **In Vitro Hatching of Oncospheres of *Hymenolepis diminuta*.** M. VOGE AND A. K. BERNTZEN, University of California, Los Angeles.

Oncospheres of *Hymenolepis diminuta* were hatched in vitro using extracts of the beetle *Dermestes vulpinus* in Tyrode's solution. Upon the addition of this extract, hatching occurs in 15 to 30 minutes after mechanical breakage of the egg shell. Oncosphere activity appears essential for successful hatching.

Various methods were used and solutions tested to determine the steps of the hatching process. The structure of the egg was studied in relation to hatching. It was observed that there were, in addition to the egg shell, four membranes enclosing the oncosphere.

During the process of hatching, friction is required for breaking of the egg shell and

rupture of the underlying membrane. The vitelline membrane apparently is digested or dissolved by substances present in the host, while the oncosphere coat and membrane rupture only with prolonged oncosphere activity, whether or not the vitelline membrane has disappeared. Extracts of *Dermestes vulpinus* prepared with distilled water or normal saline solution instead of Tyrode's do not bring about hatching. Extracts of *Tenebrio molitor* in Tyrode's solution, or Tyrode's solution alone, will not elicit hatching.

131. **In Vitro Hatching of Oncospheres of** *Hymenolepis citelli* **and** *Hymenolepis nana.* A. K. BERNTZEN AND M. VOGE, University of California, Los Angeles.

Oncospheres of *H. citelli* were hatched successfully in extracts of the beetle *Dermestes vulpinus.* Eggs were concentrated by a sugar flotation, instead of the zinc sulphate method which appears to inhibit hatching. Solutions of known concentrations and composition were prepared of trypsin, pancreatin, bile salts and Tyrode's, in various combinations. Hatching did not occur in any of the solutions containing bile salts. It was found that there was a correlation between the composition of the solution and the percentage hatched.

Oncospheres of *H. nana* were hatched in extracts of *Dermestes vulpinus.* Other solutions containing trypsin, pancreatin and bile salts, in Tyrode's, either singly or in combination, did not activate oncospheres or induce hatching. Activation on oncospheres was achieved by adding urea to the above solutions. Activation and hatching was achieved with urea in Tyrode's solution.

132. **The Importance of Coyotes** (*Canis latrans*) **in the Maintenance of Sylvatic Echinococcosis: Preliminary Observations.** JOHN C. HOLMES, University of Alberta, Canada.

A search of the literature has revealed only three well-authenticated records of *Echinococcus granulosus* in coyotes. Sweatman (1952, Can. J. Pub. Hlth. **43**: 480–486) reported an infected coyote taken in Ontario and another taken at Banff, Alberta. Miller (1953, Can. Med. As. J. **68**: 423–434) reported an infected coyote from Anahim Lake, B. C. Despite the paucity of published records of *E. granulosus* from coyotes, the latter have been suggested as important hosts in the maintenance of sylvatic echinococcosis on several occasions (Cowan, 1948, J. Wildl. Mgt. **12**: 105–106; Butler and Grundmann, 1954, J. Parasit. **40**: 440–443; Harper, et al, 1955, Tr. N.A. Wildl. Conf. **20**: 198–208; and others).

Two of 15 coyotes taken at Banff, Alberta, during the winter of 1959–1960 were found infected with *E. granulosus.* Tapeworms from one of them were compared with *E. granulosus* from wolves collected in west-central Alberta. The tapeworms from the coyotes were smaller than those from the wolves, and a smuch smaller proportion (less than 5 percent, as opposed to over 40 percent of those from the wolves) contained normally developed ova. These results indicate that *E. granulosus* can mature in coyotes, and, in combination with the epidemiological evidence of other workers, suggest that coyotes may be locally important in the maintenance of sylvatic echinococcosis.

The author is indebted to Mr. D. Flook, Canadian Wildlife Service, for making the coyotes available. This study was supported, in part, by General Research Grant No. 244 from the University of Alberta.

133. **Effects of Natural Stresses and of Cortisone Upon Acquired Resistance to** *Hymenolepis nana* **in Mice.** C. J. WEINMANN AND A. H. ROTHMAN, Rice University, Houston, Texas.

Host resistance to reinfection by the direct route elicited by a primary egg infection is cortisone-sensitive, both during the induction phase and after well established. Cortisone acetate in doses as low as 0.05 mg per mouse for 1 week may allow massive reinfection to occur when the primary adult worms are present in the gut. After cessation of cortisone treatment (0.5 mg daily for 1 week) mice recover the ability to resist reinfection within 4 to 6 days.

Observations and experiments dealing with the effects of cold, hunger, and fighting upon acquired resistance will be discussed. (This investigation was supported in part by a U. S. Public Health Service Grant No. E-1384.)

134. **Continuity of Cells From Oncosphere to Early Cysticercoid in the Development of** *Hymenolepis diminuta* **(Cestoda: Cyclophyllidea).** R. E. OGREN, Dickinson College, Carlisle, Pa.

Cellular structure of the infective oncosphere becomes the basic morphology of the early cysticercoid, here called the "post-hexacanth." The following were identifiable in both periods of oncosphere life: Six hooks, binucleate medullary center, collar myoblasts attached to the

lateral hooks, a group of small anchor cells holding the medullary center to the cortex, two lacunae and the epidermal glands. The glands, contractile in nature, were composed of four groups of cells. Small lobed portions near the hooks each contained 2 to 4 cells. Larger comma-shaped portions, situated along the sides of the body, each contained 6 to 8 cells. The epidermal gland area and its cells were identifiable during 48 hours of development in the hemocoele of *Tribolium confusum*. The first germinative cells arose in the area of the epidermal gland and were considered to be the same cells that formed the gland of the infective oncosphere. During the extended prophase of the germinative cells, their nucleoli enlarged and abundant cytoplasmic RNA accumulated. Early cysticercoid development was interpreted as a latent period during which oncosphere cells reorganized into germinative cells. Their mitotic divisions will later form embryonic mesenchyme. A germinative function was demonstrated for the epidermal gland.

135. *Cycloskrjabinia taborensis* **(Loewen, 1934) and Systematics of the Cestode Family, Anoplocephalidae.** HORACE W. STUNKARD, The American Museum of Natural History, New York.

A second infection of the bat, *Lasiurus borealis*, by the cestode *Cycloskrjabinia taborensis*, is reported. The taxonomy of the Anoplocephadiae is reviewed. The erection of the family Avitellinidae by Spassky (1950) and of the genus *Vampirella* by Spassky (1954) violate the International Rules of Zoological Nomenclature and both are invalid. *Mathevotaenia* Akhumian, 1946, is suppressed as a synonym of *Atriotaenia* Sandground, 1926. With the suppression of *Sobolevina* Spassky, 1951, as a synonym of *Ascometra* Cholodkowsky, 1912, and the removal of *Inermicapsifer* Janicki, 1910, and *Thysanotaenia* Beddard, 1911, from the Linstowiinae to the family Davaineidae by Baer and Fain (1955), the family Linstowiidae is reduced to the subfamily Linstowiinae. The suppression of *Skrjabinotaenia* Akhumian, 1946 as a synonym of *Catenotaenia* Janicki, 1904, by Wolfgang (1956) leaves the family Catenotaeniidae Spassky, 1950, with a single genus, and since the worms are clearly anoplocephalid cestodes, the group merits no more than subfamily status. On both morphological and bionomic grounds the family Anoplocephalidae is maintained with four subfamilies: Anoplocephalinae R. Blanchard, 1891; Catenotaeniidae Spassky, 1949; Linstowiinae Fuhrmann, 1907 and Thysanosomatinae Fuhrmann, 1907. There is at present no virtue in attempts to elevate these subfamilies to family status in a superfamily or suborder. When more information is available, it may be feasible to recognize suprafamilial relations in the Cyclophyllidea.

136. *Sparganum sebago*, **Incidence and Location in Host Fishes.** MARVIN C. MEYER AND ROLF VIK, University of Maine, Orono.

Diphyllobothriid larval studies in relation to their host require an accurate determination of their location as well as the total burden. In investigations involving *Sparganum sebago* Ward, 1910 (Bull. U. S. Bur. Fish. **28**: 1151–1194), in salmonids and American smelt, the conventional procedure of teasing them from the site with insect needles inserted in dowel handles was found to be inordinately tedious, time-consuming and quantitatively unreliable with respect to the total number present. Since the larvae are tenaciously entwined in the host tissue, they are often injured upon removal. Also recovery is limited to those situated near enough to the surface of the organ involved, so as to be detectable from the surface examination; those situated more deeply may escape notice. Results show that even with most painstaking efforts only about one-half of the larvae present are detected when inspection is limited to surface examination.

A modification of the pepsin-HCl digestion technique, originally employed for the recovery of *Trichinella* larvae and relatively recently extended to the recovery of metacercariae, proved a very useful method for the recovery of *Diphyllobothrium* plerocercoids from fresh fish. In addition to involving considerably less time, practically 100 percent recovery results; and, if organs are digested separately, the incidence of infection in each can be determined reliably. After several hours in the artificial digest the host tissue is macerated and the freed larvae are very active, thus facilitating their recovery. Larvae settle to the bottom and the supernatant is decanted; a rinse or two in water facilitates the examination.

Results of examinations of different species of fish infected with these plerocercoids, and the location in the hosts will be presented. (Supported in part by a grant (E-2759) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.)

137. **The Life History of *Proteocephalus parallacticus* MacLulich, 1943, from Lake Trout.** R. S. FREEMAN, Ontario Research Foundation, Canada.

Egg-bearing *Proteocephalus parallacticus* occurred in *Salvelinus namaycush* (Walbaum) from late May until early September in Algonquin Park, Ontario. A number of the six common species of planktonic copepods from these waters were placed with eggs of these worms, and plerocercoids completed development in *Cyclops bicuspidatus*, Claus, 1857, and *C. vernalis* Fischer, 1853; the former probably is the only important intermediate host. Plerocercoids required approximately 50 days to complete development at 15 C; abnormal development was common at higher temperatures. A transport host may be utilized for transmission in some waters, although a second intermediate host apparently is not obligatory.

138. **Structure and Development of Paruterine Organs in *Avitellina centripunctata* (Rivolta, 1874).** A. M. NADAKAL, Immaculate Heart College, Los Angeles.

Studies made with the help of serial sections indicated that paruterine organs in *A. centripunctata* are primarily of uterine rather than of parenchymatous origin. This finding is in general agreement with the observation made by Gough (1911, Quart. J. Micr. Sci. **56**: 317–383) in *A. goughi*. The uterus in *A. centripunctata* is a simple sac like structure containing eggs mostly in the posterior and lateral regions. The appearance of myoblasts along the anterior walls of the uteri marks the initial stage in the development of paruterine organs. The anterior walls of the uteri gradually bulge out in the form of a pouch augmented by the growth of fibrous lamellae which are abundant in the anterior half of the uteri. The growth of the uterine lamellae into the pouch results in the formation of a number of longitudinally disposed elongated chambers, which receive the eggs enclosed in fibrous capsules. During the earlier stages the paruterine organs are somewhat pear-shaped, but they assume a spherical shape as full development is reached. They remain attached to the original shriveled up uteri at the posterior border by a fairly wide neck. Slightly alternate in position in successive proglottids, the paruterine organs, and their egg-capsules, each enclosing 4 to 6 eggs, measure 380 to 390 microns and 70 to 78 microns in diameter respectively. A single paruterine organ with 10 to 15 egg-capsules usually contains on an average 70 to 90 eggs indicating the poor egg-productivity of this species as compared with some other anoplocephaline cestodes like *Moniezia*. Simultaneous with the development of paruterine organs, there appears an assemblage of myoblasts just anterior to the uteri. These represent the anlagen of the fibrous pads characteristically found in these worms.

139. **Frequency and Seasonal Fluctuations of Infections of Sheep with Certain Helminth Parasites in Northern India.** A. M. NADAKAL, Immaculate Heart College, Los Angeles.

During a 3-year period extending from 1950 to 1953, a total of 261 digestive tubes of sheep obtained from slaughter houses in the vicinity of University of Aligarh, India, were examined for various helminth parasites. Trematodes of the family, Paramphistomatidae, were noticed in approximately 5 percent of the cases; nematodes of the genera, *Bunostomum* Ancylostomidae) and *Oesophagostomum* (Strongylidae) in 50 percent of the cases, *Haemonchus* (Trichostrongylidae) in 80 percent of the cases; tapeworms of the genera *Stilesia*, *Moniezia*, and *Avitellina* (Anoplocephalidae) in 80, 50, and 30 percent of the cases respectively. Multiple infections occurred much more frequently than single ones, and very few worms were found in young hosts.

During the months of August and September immature tapeworms were encountered in the collection. Again, in February and March there was the incidence of young worms. This indicates that there are mainly two periods of fresh infections of the hosts with these cestodes, possibly centered around the months of July and August in the first instance, and December and January in the second. The periods of fresh infections seem to be correlated with rains and the subsequent appearance of vectors such as the oribatid mites on the blades of grasses facilitating their ingestion by the definitive hosts during grazing. Available data suggest that the number of infections of hosts with the tapeworms fluctuate considerably reaching a peak in October and November, and another peak in February. The 'peaks' and 'lows' were most pronounced in the case of the tapeworm genus *Stilesia*.

140. **Antigenic Analysis of *Hymenolepis* Extract by Gel Diffusion.** R. M. COLEMAN, AND N. FOTORNY, Russell Sage College, Troy, New York.

From preliminary studies, involving the gel diffusion technics of Oudin and Ouchterlony, it has been observed that whole worm extracts of *Hymenolepis nana* gives four groups of precipitation bands with its homologous rabbit antiserum prepared in intramuscular injection. A similar number of groups was observed with pooled sera from mice following a 3-week period of infection (egg induced). No bands were formed when the extract was reacted against normal rabbit or mouse serums.

Horizontal six column diffusion plates designed by Wilson (1958, J. Immunol. **81**: 317–330) along with a variety of three and five component plates of his design (not published) were employed. The plates were constructed of plexiglass by G. Eidt and prepared with the technical assistance of W. Farris, Jr. (Wayne State University, College of Medicine). Glass filtered, 0.75 percent Bacto Certified Agar (Difco) containing 0.85 percent NaCl, 0.01 percent Merthiolate and adjusted to pH 7.2 was used as the gel. Reactants, in equivalent concentrations, were placed in wells previously rinsed with 0.3 percent agar. The volumes of reactants used were 0.2 ml for the column plates and from 0.5 to 0.8 ml for the three and five component plats. Plates were covered with lids, sealed with masking tape and developed at 21 C. Greater sensitivity was observed with the double diffiusion plates as compared with single diffusion plates. The minimum reaction time for this system was 24 hours for the DD plates and the effective reaction time was observed to be 2 weeks. Plates were observed daily by means of a fluorescent light box. Some plates were refrigerated at 4 C for 2 to 5 days, following development at room temperature, which appeared to make the bands more distinct. Plates were rinsed and covered with distilled water for photography with an enlarger. In some instances 0.006 percent Orange G dye was added to the distilled water as described by Wilson (loc. cit.). (This investigation was supported, in part, by a research grant, G 14342, from the National Science Foundation and by grants from the Sigma-Xi-Resa Research Fund and Russell Sage College.)

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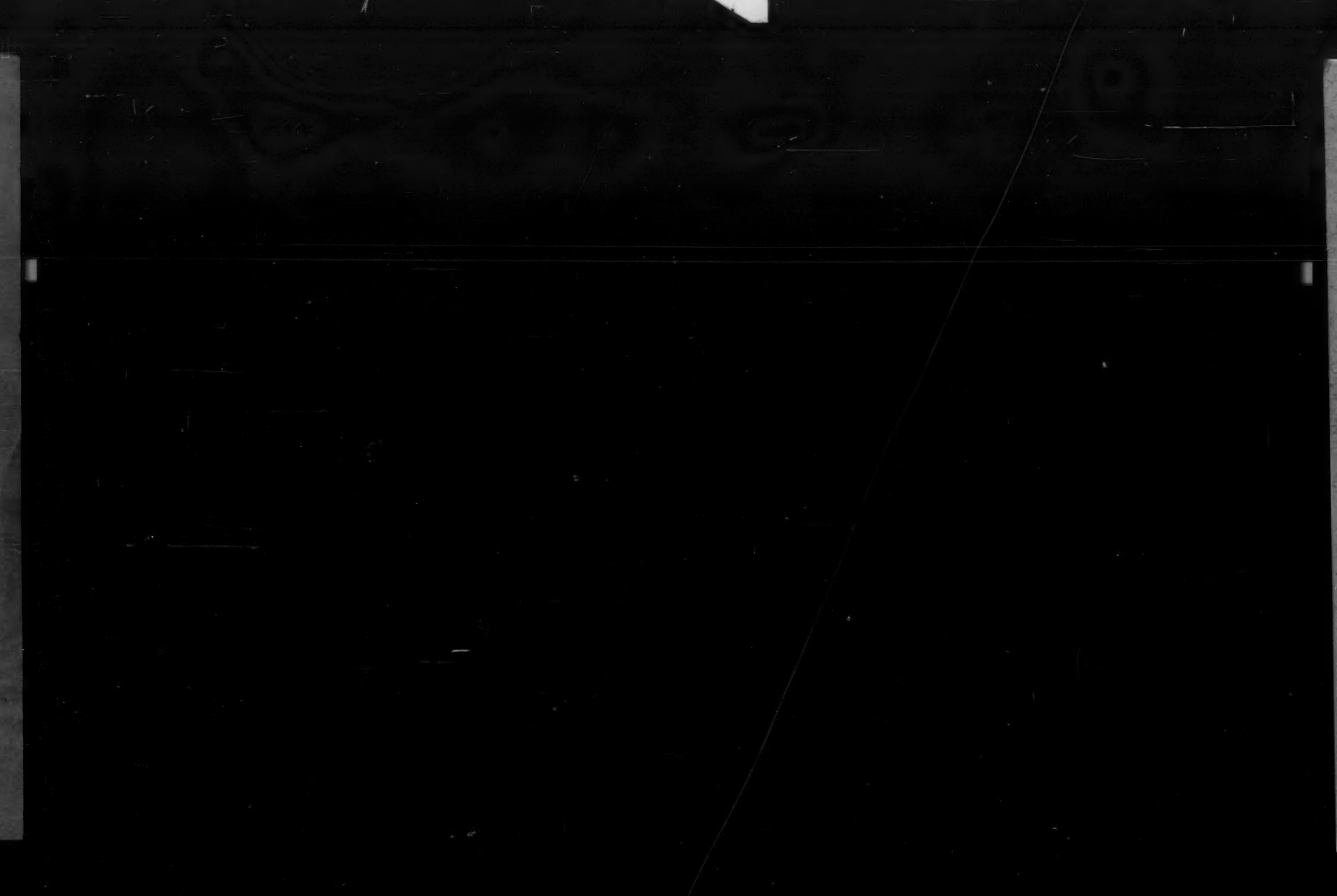
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